Cell cycle

Cell cycle model of regenerating hepatocytes in mammals

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Dedicated to my parents Ms. Suman Chauhan and Prof. K.P.S. Chauhan

Abstract

Mathematical models have been used to study the cell cycle for the last 50 years. Now it is well established that cell replication is a controlled process with sequential and timely activation and degradation of cyclins leading to swift transitions between the phases of the cell cycle. The essential achievement in identifying the key components and in dissecting the mechanisms of the cell cycle circuitry has been attributed to the simultaneous use of model systems like yeast, frogs, sea urchin, starfish, and flies. Present understanding of the cell cycle needs to be extended to investigate whether those findings also apply to mammalian in-vivo models like mice. Some mammalian cell cycle models exist focusing on specific check points or transitions, but there is no integrated model yet where the cell cycle is induced after injury in the mammalian cells.

Liver regeneration is one of the most synchronised cell proliferation phenomenon, where 95% of the cells simultaneously enter cell cycle after being induced by injury. Therefore cell cycle in regenerating livers was chosen as the model system. Focusing on how injury induced pro-inflammatory signals *prime* the cells in G1 phase and consequently both cytokine and growth factor induced pathways lead to further cell cycle progression, the G1-S phase transition was modeled. The model was further extended to mitotic events leading to the all-or-none G2-M transition and mitotic exit. I focussed on the emerging role of Cdh1 in the mammalian cell cycle. Cdh1 is known to play a key role in maintaining quiescence during G1. The role of Cdh1 in the G2 delay was further investigated. Cdh1 was suggested to be at the core of the cell cycle machinery controlling cyclin dynamics.

This model is an attempt in understanding core machinery of the mammalian cell cycle. Better understanding of the cell cycle control system in mammalian cells would enable studying cell physiology in a larger context of response to the environmental changes and heterogeneous cell proliferation at the tissue level. This leads to the major goal of cell cycle modeling in understanding perturbations of the human cell cycle machinery which lead to diseases like cancers.

Zusammenfassung

Während der letzten 50 Jahre wurden mathematische Modelle zum Studium des Zellzyklus verwendet. Nach dem heutigen Verständnis ist die Zellreplikation ein kontrollierter Prozess aus sequentieller und zeitlich koordinierter Aktivierung und Abbau von Zyklinen, die einen schnellen Übergang zwischen den Zyklusphasen ermöglichen. Dabei ist der Erfolg bei der Ermittlung der wichtigsten Komponenten und Aufgliederung der Schaltmechanismen im Wesentlichen auf die gleichzeitige Anwendung von Modellsystemen wie Hefe, Frosch, Seeigel, Seestern und Fliege zurückzuführen. Das heutige Verständnis des Zellzyklus muss erweitert werden, um zu überprüfen ob die Erkenntnisse auch auf in-vivo Modelle von Säugetieren wie der Maus zutreffen. Es existieren solche Modelle, die sich auf spezifische Kontrollpunkte oder Übergänge konzentrieren, allerdings noch kein integriertes Modell, in dem der Zellzyklus durch eine Verletzung im Säugetier induziert wird.

Das Modellsystem der Leberregeneration bei Nagern wurde gewählt, da es sich durch das am höchsten verbreitete Phänomen der Synchronisation der Zellproliferation auszeichnet. Hierbei treten 95% der Zellen nach einer Verletzung gleichzeitig in den Zellzyklus ein. Mit dem Fokus auf die Frage, wie die Zellen durch pro-inflammatorische Signale nach Verletzungen ins *Priming* in der G1 Phase eintreten, gingen wir in einen durch Zytokine und Wachstumsfaktoren induzierten Säugetier-Zellzyklus über. Dank der gut untersuchten G1-S-Phase der Leberregeneration konnten wir die nachgelagerten, durch pro-inflammatorische Zytokine und Wachstumsfaktoren induzierte Signalwege modellieren, die nach einer Verletzung zum G1/S- Übergang in der Leber führen. Weiterhin wurden mitotische Ereignisse modelliert, die zum Alles-oder-Nichts G2/M Übergang und dem mitotischen Ausgang führen. Wir konzentrieren uns auf die vielversprechende Funktion von Cdh1 in der Zellzykluskontrolle, welches bekanntlich eine Schlüsselrolle in der Aufrechterhaltung der Ruhephase während der G1 Phase spielt. Weiterhin haben wir dessen Rolle bei der Verzögerung der G2 Phase untersucht. Wir vermuten eine zentrale Rolle von Cdh1 im Zellzyklus durch die Kontrolle der Dynamik der Zykline.

Das Modell ist ein Versuch, die Kernmechanismen der Zellzykluskontrolle bei Säugetieren zu verstehen. Besseres Verständnis der Mechanismen in der Säugetierzelle würde das Studium der Zellphysiologie im größeren Zusammenhang der Antwort auf Umweltveränderungen und der heterogenen Zellproliferation auf Gewebeebene ermöglichen. Diese Schritte führen zum großen Ziel der künftigen Zellzyklusmodellierung im Hinblick auf Störungen der humanen Zellzyklusmaschinerie, welche zu Krankheiten wie Krebs führen.

Contents

1 Liver regeneration

Synopsis

Liver regeneration after surgical resection is one of the most studied models of cell, organ, and tissue regeneration. The complexity of the signaling pathways initiating and terminating this process have provided paradigms for regenerative medicine. Many aspects of the signaling mechanisms involved in hepatic regeneration are under active investigation. The purpose of this chapter is to introduce regeneration phenomena observed in rodents giving insights into the signals controlling the proliferation, function and structure of the liver during liver regeneration.

1.1 The liver

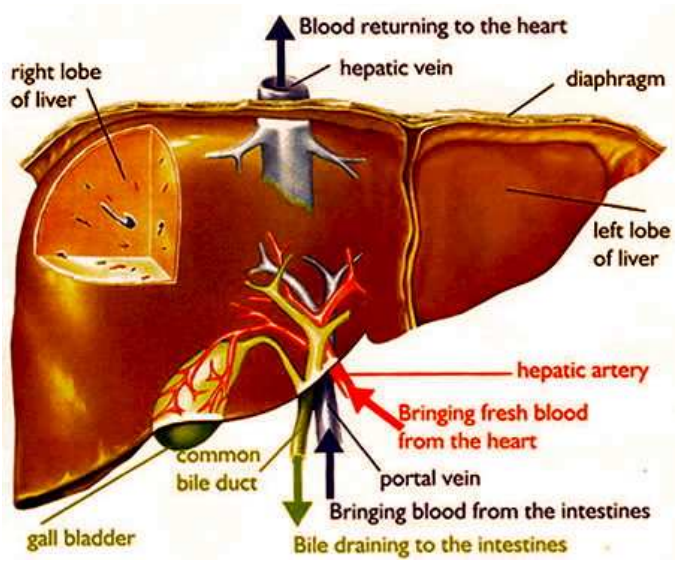

Figure 1.1:
Liver anatomy. Liver consists of two main lobes: left lobe and right lobe. Blood from heart and intestines is supplied to liver through the portal vein and purified blood leaves through the hepatic vein. Upon injury the cell proliferation first begins in the cells that surround the portal vein of the liver lobule and then proceed towards the hepatic artery.

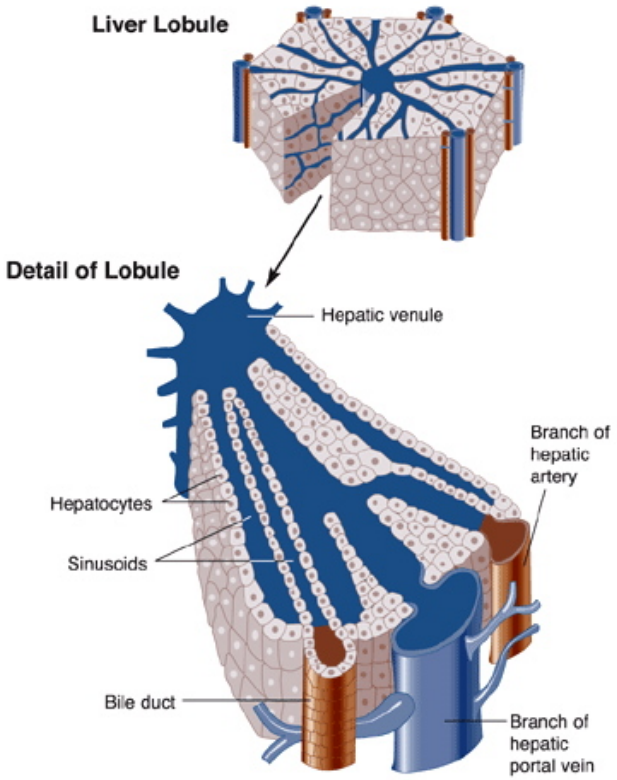

Figure 1.2:
Liver lobule. Lobule is the functional unit of liver. Hepatocytes are the main cell types in the lobules which arrange themselves into hepatic plates. Sinusoids surround these hepatic plates and maintain the flow of blood between branches of portal and hepatic veins supplying essential nutrients to the cells.

The liver is an important organ of the body that has a central role in metabolic homeostasis, as it is responsible for the metabolism, synthesis, storage and redistribution of nutrients, carbohydrates, fats and vitamins. The liver produces large numbers of serum proteins including albumin, acute-phase proteins, enzymes and cofactors. Importantly, it is the main detoxifying organ of the body removing wastes and xenobiotics by metabolic conversion and biliary excretion. There are two distinct sources that supply blood to the liver: 1) oxygenated blood flows in from the hepatic artery; 2) nutrient-rich blood flows in from the hepatic portal vein (Fig. 1.1).

At the central area the common bile duct, portal vein, and hepatic artery enter the liver. Branches of the hepatic artery and portal vein guide blood to the periportal regions of the lobules. From there, it flows through microvessels, the sinusoids, along hepatocyte columns that are lined with endothelial cells (generally known as sinusoidal cells), and drains into the central vein. The liver consists of two main lobes. These lobes are organized in repetitive functional units called liver lobules, which besides its main constituents, hepatocytes, consists of sinusoidal endothelial cells, Kupffer, stellate, and bile duct cells. Upon injury the cell proliferation first begins in the cells that surround the portal vein of the liver lobule and then proceed towards the central

vein. The complex lobule architecture ensures a maximal exchange area between blood and hepatocytes in healthy liver (Fig. 1.2). In liver disease, such as hepatocellular cancer, the contact surface between hepatocytes and sinusoidal cells decreases and contributes to compromised liver function (Hoehme et al., 2010) .

1.2 Liver regeneration

Figure 1.3:
Liver regeneration legend: Prometheus revisited. Liver is the main detoxifying organ of the body. Thus nature provides it with a remarkable regeneration capacity against injury from ingested toxins. First depiction of liver regeneration capacity can be traced back in the Greek myth. Prometheus was punished by Gods of Olympus for stealing the secret of fire from them. A portion of his liver was eaten up by an eagle daily, which regenerated overnight and provided eagle with eternal food and Prometheus with eternal pain (Taub, 2004).

Being the main detoxifying organ of the body, it is quite susceptible to get damaged by ingested toxins. In order to maintain its architecture and function, nature provides it with a remarkable capacity to regenerate after injury by counterbalancing the cell death with compensatory cell division. Earliest recognition of its extraordinary regenerative capacity can be found in Greek myth of Prometheus. Gods of Olympus punished prometheus for stealing the secret of fire from them. A portion of his liver was eaten up by an eagle daily, which regenerated overnight, thus providing eagle with eternal food and Prometheus with eternal pain (Fig. 1.3). Although adult hepatocytes are long lived and normally do not undergo cell division, they maintain the ability to proliferate in response to toxic injury and infection.

Remarkable capacity of liver to regenerate after injury and to adjust its size to match its host has intrigued scientists since many years. The regenerative process is compensatory because the

size of the resultant liver is determined by the demands of the organism, and, once the original mass of the liver has been re-established, proliferation stops. Adding to its adaptive capacity, in some cases, transplanting liver from a baboon to a human, caused liver to grow to the size of the human liver and transplanting liver from a large dog to a small dog led to loss of liver mass until it reached the size appropriate to the small dog (Michalopoulos and DeFrances, 1997). There are various central questions regarding the process of liver regeneration. What triggers the process of liver regeneration? How the size and function of liver is maintained during regeneration? What turns off the phenomenon once the liver mass is reconstituted? Understanding the process might assist in treatment of serious liver diseases and may also find implications for certain types of gene therapies.

1.3 Experimental animal models of liver regeneration

Most studied experimental models of liver regeneration are those of rodents (mouse and rat). Studies with hepatic resections in larger animals (dogs and primates) and humans have established that the regenerative response is proportional to the amount of liver removed. Studies on transplantation of liver from other bigger animals to humans (Francavilla et al., 1992; Starzl et al., 1993) demonstrate that liver mass is precisely regulated and that signals from the body can have negative as well as positive effects on liver mass until the correct size is reached. Type of liver injury inflicted to the animal can be classified into two types: 1) Partial hepatectomy and 2) Cell necrosis .

Partial hepatectomy Partial hepatectomy is the surgical removal of a part of liver. Bucher and Swaffield (1964) show that the extent of hepatocyte replication in the regenerating liver of adult rats is proportional to the amount of tissue resected for resections involving 40-70% of the liver. Removal of 30% of the liver lies below this threshold and does not elicit a clear wave of DNA replication. Liver regeneration phenomena is most clearly shown by the 70% partial hepatectomy model in rodents, which was pioneered by Higgins and Anderson in 1931 (Higgins and Anderson, 1931) . It is a simple operation (partial hepatectomy, PH) in which two-thirds of the liver of a rat is removed. Specific liver lobes are removed intact, without damage to the lobes left behind. The residual lobes enlarge to make up for the mass of the removed lobes, though the resected lobes never grow back. The whole process lasts 5 to 7 days.

Cell necrosis In this model hepatocytes are directly damaged and thereby induced to undergo necrosis, similar growth-factor- and cytokine mediated pathways are activated as occurs after partial hepatectomy (Dabeva and Shafritz, 1993). Proliferation of hepatocytes is also involved in the liver regeneration that occurs after massive hepatocyte necrosis, or apoptosis that is induced by hepatic toxins such as CCl_4 or systemically introduced Fas ligand, but the cell-cycle response

is not as synchronized (Fausto, 1999). As expected, there are also significant changes in liver architecture during liver regeneration, both after partial hepatectomy and liver necrosis.

1.4 Patterns of DNA synthesis and gene expression during regeneration

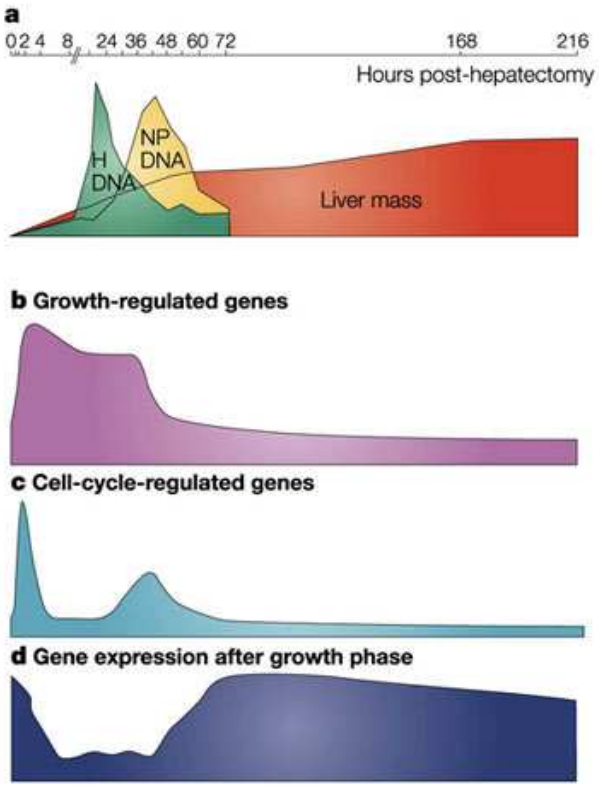

Figure 1.4:
Patterns of DNA synthesis and gene expression during rat liver regeration. (a) After partial hepatectomy, DNA synthesis in hepatocytes (H; green) peaks around 24 hours, whereas DNA synthesis in the non-parenchymal cells (NP; yellow) peaks around 36–48 hours. Re-accumulation of liver mass (red) is complete within a week. (b) The induction pattern of gene expression for growth-regulated genes, such as -actin. (c) The induction pattern of gene expression for cell-cycle-regulated genes, such as insulin-like-growth-factor-binding protein-1 (IGFBP1). (d) Some genes, such as that encoding the isoform of CCAAT-enhancer-binding protein (C/EBP), are downregulated during the period of maximal growth and are re-expressed after the growth phase has occurred (Taub, 2004). Note that in mice DNA synthesis peaks 12–16 hours later compared to rat (Weglarz and Sandgren, 2000)

Cell division is rarely seen in hepatocytes in the normal adult liver, as these cells are in the G0 phase of the cell cycle (Michalopoulos and DeFrances, 1997; Webber et al., 1994). However,

after partial hepatectomy approximately 95% of hepatic cells, which are normally quiescent, rapidly re-enter the cell cycle. In the rat liver, the rate of DNA synthesis in hepatocytes begins to increase after about 12 hours as they enter the S phase of the cell cycle and peaks around 24 hours (Fig. 1.4). However, the induction of DNA synthesis occurs later in the non-parenchymal cells (at 48 hours for Kupffer and biliary epithelial cells, and at 96 hours for endothelial cells). Subsequent levels of DNA synthesis in hepatocytes are lower, as complete restoration of liver mass requires an average of 1.6 cycles of replication in all cells. By comparison, the peak in DNA synthesis in mice occurs later (36–40 hours after partial hepatectomy) and varies between strains. The onset of DNA synthesis is well-synchronized in hepatocytes, beginning in cells that surround the portal vein of the liver lobule and proceeding towards the central vein. The incidence of mitosis (M phase) is lower than is predicted on the basis of the number of hepatocytes that undergo DNA synthesis, and the ploidy of hepatocytes and percentage of binucleate cells increases with successive rounds of DNA synthesis, which ultimately limits further regeneration. Most of the increase in liver mass has occurred by 3 days after partial hepatectomy and mass restoration is complete in 5–7 days (Taub, 2004).

Changes in gene expression associated with regeneration are observed within minutes of hepatic resection (Fig. 1.4). Growth regulated genes demonstrate elevated expression throughout the entire growth phase and expression returns to normal after about three days. Cell cycle regulated genes show a sharp peak of expression that coincides with the G1 phase of hepatocyte cell cycle, including the first round of replication and a second smaller round of replication that occurs 48 hours after 2/3 PH. In this way a cell cycle regulated cascade of gene and protein expression allows cells to progress through the G1 phase of cell cycle. Ultimately, changes in the levels of cyclins and their regulatory kinases allow for transition through the late phases of G1 (D-type cyclins) into S phase (Cyclin E) (Taub, 2004).

1.5 Autonomy vs circadian control of liver regeneration

The extent and timing of liver regeneration are known to vary according to circadian rhythms (Barbason et al., 1995); a recent study has identified a mechanism by which these rhythms control hepatocyte proliferation after PH. In these experiments, the peak of DNA replication after PH in mice always occurred 36 hours after the operation, regardless of the time of the day at which the procedure was performed. The entry of cells that had replicated their DNA (G2 cells) into mitosis, however, always occurred at the same time of day. This finding suggests that a circadian clock controls the G2/M transition (Matsuo et al., 2003b).

On the other hand, the timing of DNA replication, which is not under the control of circadian rhythms, appears to be an intrinsic property of hepatocytes. Rats and mice differ in the timing of DNA replication after PH, which is 12 to 16 hours earlier in rats. Weglarz and Sandgren (2000) transplanted rat hepatocytes into the livers of mice after PH and found that rat hepatocytes replicated earlier than mouse hepatocytes in the resultant chimeric liver. These results indicate

that the timing of hepatocyte DNA replication after PH is an autonomous process, primarily guided by intrinsic signals .

1.6 Metabolic pathways and liver regeneration

Liver regeneration after PH is a perfectly calibrated response whose apparent sensor is the body's requirement for liver function. The increased metabolic demands imposed on the liver remnant after PH are likely connected with activation of the machinery directly involved in DNA replication. mTOR (mammalian target of rapamycin) is part of a complex that senses nutrient or energy status, and also integrates growth factor signals. In the regenerating liver, rapamycin – a pharmaceutical agent that is known to block hepatic regeneration – inhibits the activation of Cyclin D through the inhibition of mTOR, thereby preventing progression through G1 and entry into the DNA-synthesis phase of the cell cycle (Taub, 2004). The mTOR complex may regulate liver regeneration by modulating cell growth and proliferation in response to the energy demands of the remaining liver, given that rapamycin, an inhibitor of mTOR, inhibits DNA replication after PH.

Several of the liver-restricted immediate-early genes encode enzymes and proteins that are involved in regulating the gluconeogenic response of the liver. Gluconeogenesis results in the net production of glucose by the liver, which increases the serum glucose level and can also be used to produce glycogen, glycoproteins and other sugars. The induction of gluconeogenic genes by partial hepatectomy represents an adaptive response of the liver whereby the remaining third of the liver compensates to produce sufficient glucose for the whole organism. Liver-specific transcription factors have an important role in determining liver-specific functions, including the level of glucose production, by regulating the expression of genes that encode liver-specific enzymes (such as metabolic enzymes) and liver-specific secreted proteins (such as albumin). The adaptive response of the liver during regeneration, which allows for the maintenance of metabolic homeostasis, is accomplished by the interplay between different sets of transcription factors. Specifically, this involves those transcription factors that are induced by the regenerative response, and those that are normally expressed in the liver, to regulate the differentiated functions of the hepatocyte (Taub, 2004).

Expression of many liver-specific genes – such as those which encode IGFBP1, glucose 6-phosphatase and α-fibrinogen is regulated in the basal state by hepatic nuclear factor-1 (HNF1), which is a liver-specific transcription factor. Transcriptional activity of HNF1 is upregulated during liver regeneration, which is accomplished by binding of HNF1 to the growth-induced transcription factors STAT3 and AP1. So, together, these two types of transcription factors – growth-induced (STAT3 and AP1) and tissue-specific (HNF-1) – provide an adaptive response to liver injury and amplify the expression of hepatic genes that are important for the homeostatic response during organ repair (Taub, 2004). Such mechanisms enable the liver to maintain metabolic function, despite the loss of two thirds of its functional mass.

2 G1 and S phase of the cell cycle during liver regeneration

Synopsis

During liver regeneration, normally quiescent hepatocytes enter cell cycle. A large number of genes are involved in the cell cycle control during liver regeneration. The essential circuitry required for the process comprises cytokine and growth factor induced pathways. The innate immune system plays an important role in the initiation of liver regeneration after an induced external damage. Injured sites release cytokines which *prime* the hepatocytes to readily respond to growth factors and enter the cell cycle. This chapter summarizes the known molecular and cellular mechanisms of liver regeneration which lead to initiation of the cell cycle machinery and DNA synthesis.

2.1 Introduction

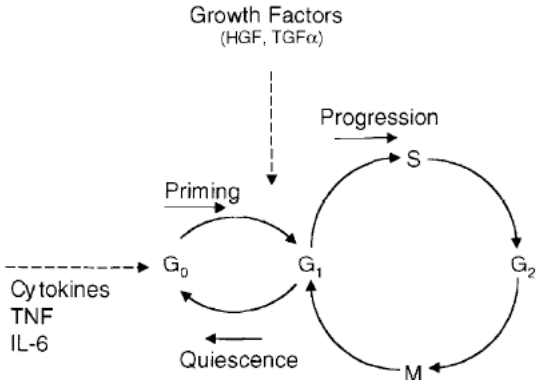

Figure 2.1:
Hepatocyte cell cycle. Hepatocyte cell cycle can be divided into two parts: *priming* and progression. Pro-inflammatory cytokines like TNF, IL-6 prime the quiescent cell to G1 phase from where cells can return to quiescence. At late G1, upon being induced by growth factors such as HB-EGF, HGF, cells commit themselves irreversibly to further progression. Growth factor HB-EGF mainly lies at the interface of *priming* and progression (Fausto et al., 1995).

In a normal adult liver cells rarely divide and are considered to be in a quiescent state, i.e., in the G0 phase of cell cycle. However, after 2/3 PH 95 % of the hepatic cells rapidly enter cell cycle (Fig. 2.1). The eukaryotic cell cycle is traditionally divided into four phases: S-G1-M-G2. S is the synthesis phase during which the DNA replicates and M is the mitosis phase during which chromosomal separation occurs and cells finally divide. S and M phase are separated in time by two gaps (G1 and G2 phases). During G1 and G2 phases cells prepare for the next phase, synthesizing needed protein and increasing their mass. The transition of quiescent cells from G0 phase to G1 phase, which is often called *priming* (Fausto, 2000), is reversible and from here cells can return to quiescence upon growth factor withdrawal. Once it crosses G1 check point cell is committed to DNA synthesis and completion of a cell division cycle (Fig. 2.1).

2.2 Cell cycle during liver regeneration

Liver regeneration is a complex process, involving activation of multiple pathways, including those induced by cytokines (e.g. interleukin-6 (IL-6), tumor necrosis factor (TNF-α)), growth factors (e.g. hepatocyte growth factor (HGF), epidermal growth factor (EGF), heparin binding growth factor (HB-EGF), transforming growth factor (TGF-α)), thyroid hormones, insulin and norepinephrine (Fig. 2.2). The entry of quiescent hepatocytes into the cell cycle can be divided into two parts: *Priming* and progression (Fig. 2.1).

1. **Priming:** During *Priming* cells exit the G0 quiescent state (G0-G1 transition) and become sensitive towards growth factors. Cells do not respond to growth factors before being primed. *Priming* is triggered by TNF-α and IL-6 released from Kupffer cells. IL-6/TNF-α elicit immediate early gene expression in hepatocytes (Fausto, 2000).

2. **Progression:** Growth factors trigger primed cells to enter DNA synthesis. Growth factors including HGF, HB-EGF help the cell to pass through G1 restriction point. Primed cells with growth factors progress through G1 phase with appearance of Cyclin D in the mid-G1 phase. Cyclin D is important for progression beyond a late G1 restriction point. Appearance of Cyclin E in the late G1 phase and early S phase and its tightly regulated expression makes it the most important marker for G1-S transition. Eventually, Cyclin D and Cyclin E levels are brought down by a family of cyclin dependent kinase inhibitors (CKIs) (Fausto, 2000).

Besides cytokines and growth factors there are many other signals from various cell types amplifying hepatocyte activity. Insulin and norepinephrine amplify the mitogenic response of EGF and HGF. Norepinephrine rises rapidly in the plasma within 1 hour after PH (Cruise et al., 1987). Norepinephrine induces secretion of EGF from the Brunner's glands of the duodenum (Olsen et al., 1985). Pancreatic islets supply insulin to the liver continually through portal vein. Insulin infusion corrects liver atrophy by a process involving hepatocyte replication (Gupta et al., 1988).

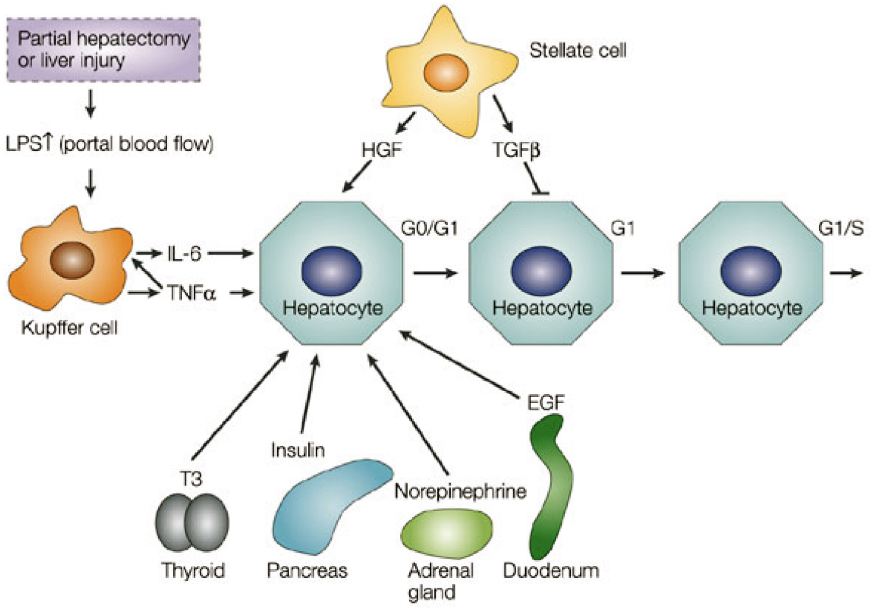

Figure 2.2:
Liver regeneration as interplay of several pathways. After liver injury, several signals are initiated simultaneously in the liver. Gut-derived factors, such as LPS, are upregulated after liver injury or hepatectomy and reach the liver through the portal blood supply. They activate hepatic non-parenchymal cells (including Kupffer cells and stellate cells) and increase the production of TNFα and IL-6. Other factors are released from the pancreas (insulin), duodenum or salivary gland (EGF), adrenal gland (norepinepherine), thyroid gland (T3) and stellate cells (HGF). Cooperative signals from these factors allow the hepatocytes to overcome cell-cycle checkpoint controls and move from G0, through G1, to the S phase of the cell cycle. This leads to DNA synthesis and hepatocyte proliferation. TGFβ signaling, which inhibits hepatocyte DNA synthesis, is blocked during the proliferative phase but is restored at the end of the process of regeneration by helping to return hepatocytes to the quiescent state (Taub, 2004).

2.2.1 Priming phase

Pro-inflammatory cytokine pathway (PICs)

The PICs induced initiation of liver regeneration: Cytokines which are small secreted proteins which mediate and regulate immunity, inflammation, and hematopoiesis. They are produced in response to an immune stimulus. Pro-inflammatory cytokine are a general term for those immuno-regulatory cytokines that favor inflammation. Tumor necrosis factor (TNF) and interleukin-6 (IL-6) are two major pro-inflammatory cytokines that are responsible for early responses. They are mainly produced by Kupffer cells. Pro-inflammatory network is initiated through the binding of TNF to its receptor TNFR1, leading to activation of NF-κB in Kupffer cells NF-κB activation results in upregulation of IL-6 transcription in Kupffer cells. IL-6 is released into the serum and activates the neighboring hepatocytes by binding to its receptor, IL-6R. Activation of IL-6R which is a complex of gp80 and gp130 subunits leads to phosphorylation of STAT3 (signal transducer and activator of transcription 3) monomers by JAKs (janus-associated

kinases). STAT3 then homodimerizes and translocates to the nucleus, where it induces transcription of a number of target genes. lL-6 is also able to signal via the Map kinase pathway (Fig. 2.3) (Taub, 2004).

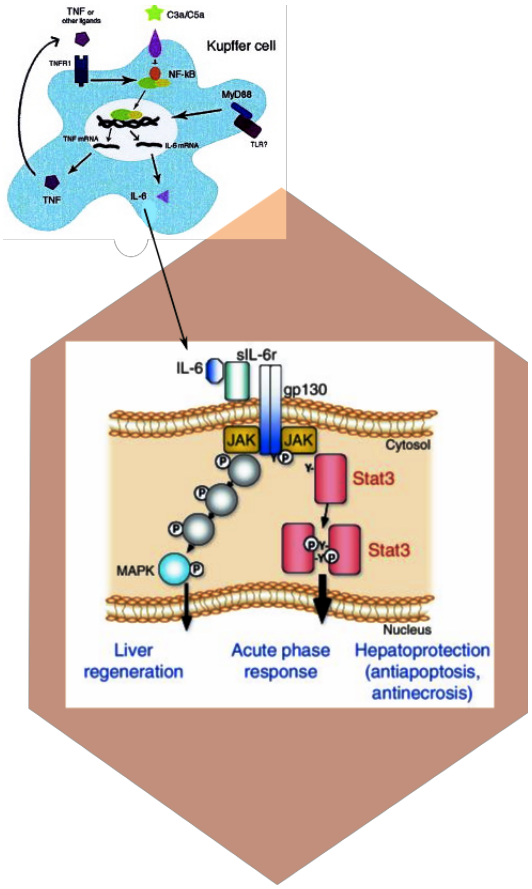

Figure 2.3:
Cytokine pathways activated during liver regeneration. The figure illustrates interactions in cytokine pathways between Kupffer cells and hepatocytes in the regenerating liver (other non-parenchymal cells also may be involved). TNF binds its receptor TNFR1 on Kupffer cells, leading to the activation of NF-κB. Components of immune system such as C3a, C5a, and MyD88 also can activate NF-κB after PH. IL-6 and TNF are both NF-κB target genes; IL-6 is subsequently released into the serum, and binds to its receptor on hepatocytes. Activation of gp130, which is one of the subunits of the receptor complex , leads to phosphorylation of STAT3 monomers by JAKs. STAT3 then homodimerizes and translocates to the nucleus, where it induces transcription of a number of target genes. In parallel with STAT3 phosphorylation, gp130 activation also leads to activation of ERK and MAPK signaling. All these signaling events lead to transcription of several genes involved in various liver regeneration processes (adapted and extended from (Fausto et al., 2006) and (Taub, 2004)).

Why PICs are important for initiation of liver regeneration Evidence for the importance of pro-inflammatory cytokines during this phase of regeneration includes (1) increase in liver mRNA and serum levels of TNF and IL-6 after PH (Akerman et al., 1992; Trautwein et al., 1996; Iwai et al., 2001); (2) activation of the transcription factors NF-κ B and STAT3 (Fitzgerald

and Kreutzer, 1995; Cressman et al., 1995); (3) inhibition of DNA replication by anti-TNF antibodies[20]; (4) blockage of liver regeneration in IL-6 and TNF receptor type I (Tnfr1) KO mice (Cressman et al., 1996; Yamada et al., 1997); and (5) correction of the defect in TNFR1 KO mice by IL-6 injection (Yamada et al., 1997).

Further evidence that cytokines are important for regeneration arises from the fact that certain cytokines have the ability to prime resting hepatocytes for cell division without PH. Hepatocytes in the normal liver are quiescent (G0 phase) and exhibit only a minimal response to potent in vitro mitogens, such as transforming growth factor alpha (TGF), epidermal growth factor (EGF), and hepatocyte growth factor (HGF). However, growth factor infusion into rats preceded by a single TNF injection induces replication in up to 40% of hepatocytes in the normal liver (Webber et al., 1998).

The precise role of IL-6 in liver regeneration has been particularly difficult to define. It has been calculated that almost 40% of the immediate early genes expressed in the regenerating liver (Su et al., 2002; White et al., 2005) may be IL-6 dependent, (Li et al., 2001) suggesting that the role of IL-6 in this process is complex. The primary function of IL-6 in regeneration was originally shown to be proliferative, as IL-6 KO mice had a striking deficit in DNA replication after PH (Blindenbacher et al., 2003; Klein et al., 2005; Wallenius et al., 2001; Sakamoto et al., 1999; Wuestefeld et al., 2003).

IL-6 production is needed only for a short period to induce hepatocyte growth, as uncontrolled synthesis would lead to continuous acute phase response and catabolism detrimental to the health of the host. The main production machinery of IL-6, Kupffer cells, are adapted to control their own IL-6 production through PGE2 (Goss et al., 1992). PGE2 is induced by IL-6 activated by Kuppfer cells(Fennekohl et al., 2000). When PGE2 is significantly elevated it starts inhibiting IL-6 in a negative feedback loop fashion (Goss et al., 1992, 1993). PGE2 negatively regulates the production of both TNF-α (Tanaka et al., 1996) and IL-6 (Callery et al., 1990; Goss et al., 1992).

Triggering the PICs: Role of components of the innate immune system Because cytokine activation participates in the initiation of liver regeneration, identifying the mechanisms that trigger the activation of this network is important. A logical candidate for a master upstream molecule is lipopolysaccharide (LPS), which is released from enteric bacteria into the portal circulation (Cornell, 1985). Indeed, Cornell (1990) found that rats with restricted production of LPS and mice that are naturally hypo-responsive to LPS (C3H/HeJ mice) have a delay in regeneration after PH. The LPS resistance of C3H/HeJ mice (Poltorak et al., 1998) was later found to be the consequence of a point mutation in the gene for Toll-like receptor 4 (TLR4), a member of a class of receptors that bind various microbial products. LPS binding to TLR4 activates multiple intracellular signaling pathways, some of which are dependent on myeloid differentiation factor 88 (MyD88), an adapter protein that mediates intracellular signals from several TLRs (Akira et al., 2001). Myd88 KO mice failed to activate TNF and IL-6 (Seki et al.,

2005; Campbell et al., 2006). STAT3 activation and expression of important STAT3 target genes, such as Socs3 and acute phase response genes, were also blocked in Myd88 KO mice after PH. Identifying the ligand and receptor that signal through MyD88 early after PH is an exciting challenge, and perhaps in doing so the mechanisms that initiate the liver regeneration cytokine cascade will be identified.

Other components of the innate immune system appear to be critical for normal regeneration as well; mice deficient in the C3 and C5 components of complement display significant deficits after PH (Strey et al., 2003). In these animals, diminished activation of the cytokine pathway is manifested by lack of increases in TNF and IL-6 levels, and in impaired NF-B and STAT3 activity. Whether and how these two aspects of innate immunity, TLR-MyD88 signaling and the complement cascade, converge to initiate cytokine signaling in liver regeneration is not clear.

2.2.2 Progression Phase

Cell cycle progression induced by growth factors The cytokine network acts at the *priming* phase of liver regeneration, which corresponds to the passage of quiescent hepatocytes into the cell cycle (G0 to G1). Cell cycle progression is then driven by growth factors, which override a restriction point in late G1. Passage from G1 to S phase is associated with retinoblastoma (Rb) phosphorylation, increased expression of the Rb family members and of Cyclin D, -E, and -A, and formation of Cyclin D and Cyclin E complexes with their cyclin dependent kinase partners Cdk4 and Cdk2 respectively (Menjo et al., 1998; Albrecht et al., 1993, 1998). Rb dependent regulation of cyclins is covered in more details in section 3.2.

HGF and the EGF receptor (EGFR) ligand family are important growth factors that drive cell cycle progression during liver regeneration (Matsumoto and Nakamura, 1992; Michalopoulos and Khan, 2005). HGF is produced by mesenchymal cells and acts on hepatocytes in a paracrine or endocrine fashion. Its effects are multiple and have been grouped into morphogenic, mitogenic, and mitogenic categories. HGF is known to be the most potent mitogenic growth factor, with meticulously maintained expression by activation and inhibition via urokinse-type plasminogen activator (uPA) and plaminogen activator inhibitor (PAI), respectively (Shimizu et al., 2001). PAI is induced by mediators of acute phase response such as IL-6 and plays an important role in early stages of liver regeneration. PAI suppresses the active form of HGF thus negatively controlling its activity. HGF expression is stimulated by several factors, which are seen to be elevated during immediate early phase including interleukines (IL-6, TNF-α) (Liu et al., 1994; Ohira et al., 1996), C/EBP-β (Jiang and Zarnegar, 1997; Liu et al., 1994) and a range of growth factors (EGF, TGFα) (Gohda et al., 1994; Matsumoto et al., 1992). Via its receptor *Met*, HGF activates many signaling molecules (Ras/Erk/MAPK, PI3K/Akt) and immediate early genes (c-jun, c-fos) (Borowiak et al., 2004). Few signal transduction molecules (for example, ERK, JNK, MAPK), transcription factors (for example AP1,C/EBP-β) and many other downstream targets seem to be shared and synergistically activated by cytokines and growth factors (Taub, 2004).

Studies of liver regeneration in mice with hepatocyte-specific deletion of c-met, the gene for the HGF receptor, were conducted (Huh et al., 2004; Borowiak et al., 2004). Borowiak et al. (2004) demonstrated that HGF/c-met signaling is essential for cell cycle entry after PH, and that it is responsible for the activation of extracellular signal-regulated kinase 1/2 (ERK1/2). In contrast, Huh et al. (2004) reported that hepatocyte c-met-deficient mice had massive mortality after PH, and thus examined the role of this pathway in other liver injury models. They conclude that HGF/c-met signaling is important in hepato-protection from apoptosis, and in facilitating healing after CCl_4 administration. The discrepancy in post-operative survival between the two reports is most likely related to the different surgical techniques used by the two groups, as noted by Borowiak et al. (2004). Until additional data become available, deciding whether HGF/c-met signaling functions primarily in mitogenesis, or whether it maintains hepatocyte homeostasis and thus facilitates cell replication, is not possible.

The family of ligands that bind the EGFR, in addition to EGF, includes TGF, heparin-binding EGF-like growth factor (HB-EGF), and amphiregulin (AR). TGF is an autocrine growth factor, both produced by and active on hepatocytes (Mead and Fausto, 1989). Although TGF has effects on cell motility and vascularization, its main effect is the stimulation of cell proliferation. Transgenic mice that overexpress TGF display constitutive hepatocyte proliferation and eventually develop cancer (Webber et al., 1994). TGF expression increases after PH in wild-type mice, but TGF KO mice have no defects in liver regeneration (Russell et al., 1996). The normal regeneration seen in these animals is likely a consequence of compensation by other EGFR ligands, although the roles of these growth factors after PH are not entirely redundant, as discussed below.

HB-EGF is an important growth factor during hepatic regeneration. HB-EGF induction is sensitive to the degree of damage (1/3 PH vs 2/3 PH) (Mitchell et al., 2005a) and other kind of damages (Temizer et al., 1992; Kiso et al., 1995, 1996), while *priming* genes like IL-6, TNF-α, c-jun,c-fos,c-myc are similarly expressed in 1/3 PH and 2/3 PH. HB-EGF expression is induced by Raf/MAPK pathway (Ellis et al., 2001; McCarthy et al., 1997, 1995), which is activated by HGF via *met* signaling, as mentioned earlier.

HB-EGF is expressed earlier than HGF and TGF after PH and appears to have a unique role in liver regeneration (Kiso et al., 1995, 2003). A 30% PH does not result in coordinated DNA replication, despite activation of the cytokine cascade (Bucher and Swaffield, 1964; Mitchell et al., 2005a). A single injection of HB-EGF 24 hours after 30% PH can override this blockage between *Priming* and cell cycle progression, eliciting a wave of DNA replication. Interestingly, this effect cannot be accomplished by similarly injecting HGF or TGF (Mitchell et al., 2005b). In addition, HB-EGF KO mice have a delay in DNA replication after 70% PH, although this deficiency is partially compensated by an earlier increase in TGF expression in these animals.

Both c-met and the EGFR are receptor tyrosine kinases, which recruit enzymes and scaffolding proteins to phosphorylated intracellular domains of each receptor. Multiple intracellular signaling pathways are thus activated, which regulate a multitude of transcription factors, initiate

translation, and regulate metabolic pathways. One mitogenic signal transduction pathway that is of particular interest, because it may integrate cytokine signals as well as growth factor signals, is the Ras-Raf-MEK cascade, which results in the activation of ERK1/2. ERK1/2 activation is correlated with hepatocyte DNA replication in vivo and hepatocyte proliferation in vitro (Talarmin et al., 1999; Thoresen et al., 2003; Li et al., 2002; Coutant et al., 2002). Moreover, growth factors such as HGF and TGF and cytokines such as TNF and IL-6 stimulate ERK1/2 activity in primary hepatocytes and hepatocyte cell lines (Argast et al., 2004; Francavilla et al., 1986; Scheving et al., 2002).

2.2.3 CKIs during liver regeneration

Assembly of cyclins with their activating partners, cyclin dependent kinases (cdks) ,and their enzymatic activity are regulated by a number of small proteins termed CKIs. To date, two different families of CKIs have been described in mammalian cells that differ in structure, mechanism of inhibition, and cdk target specificity. The Ink family of CKIs includes the tumor suppressor protein p16Ink4a, as well as p15Ink4b, p18Ink4c, and p19Ink4d, that appear to specifically target the G1 phase Cyclin D-Cdk4/Cdk6 complexes (Sherr and Roberts, 1995, 1999). The structurally and functionally distinct Cip/Kip family comprises three proteins: p21 (also known as Cip1, Waf1, Sdi1) (el Deiry et al., 1993; Harper et al., 1995; Noda et al., 1994; Xiong et al., 1993), p27Kip1 (Polyak et al., 1994; Toyoshima and Hunter, 1994), and p57Kip2 (Lee et al., 1995; Matsuoka et al., 1995). These proteins are able to bind and inhibit with different efficacy the activity of most cyclin-cdk complexes including Cdk2, Cdk3, Cdk4, and Cdk6 (Harper et al., 1995; Matsuoka et al., 1995).

A wide variety of environmental signals can regulate expression and activity of CKIs. Interestingly, either growth arrest resulting from DNA damage, cell senescence, and terminal differentiation or cell cycle entry and progression after stimulation with growth factors were accompanied by p21 gene activation (Macleod et al., 1995; Noda et al., 1994; Nourse et al., 1994; Sherr, 1994) through various transcription factors including p53 (el Deiry et al., 1993; Macleod et al., 1995). Beyond its role as a CKIs, at low stoichiometric concentrations, p21 may act as an assembly factor for active Cyclin D-Cdk4/6 complexes and could potentially function as an activator of these kinases (Cheng et al., 1999; LaBaer et al., 1997; Sherr and Roberts, 1999). In contrast to p21, expression of p27 protein generally declines in several cell types in response to mitogenic stimulation (Agrawal et al., 1995; Nourse et al., 1994). Furthermore, inhibitory activity of this protein increases by different anti-mitogenic signals, such as TGF- or by contact inhibition (Polyak et al., 1994; Poon et al., 1995).

Several reports (Dotto, 2000; Glaise et al., 1998; Macleod et al., 1995; Matsuoka et al., 1995; Sherr and Roberts, 1995) highlight the important difference that exists between cell types concerning regulation of these CKIs, expression, and role during development and differentiation. Particularly, these proteins are involved in coordinate regulation of cell proliferation and differentiation and maintenance of terminally differentiated cells in quiescent state (Zhu and Sk-

oultchi, 2001). Because of the high proliferative potential of mature hepatocytes at the adult stage, a specific regulation of CKIs is intended. Several prior studies (Albrecht et al., 1997, 1998, 1999; Ehrenfried et al., 1997; Jaumot et al., 1999; Kato et al., 1998; McIntyre et al., 1999; Pujol et al., 2000; Timchenko et al., 1997) have documented the pattern of CKIs expression in proliferating hepatocytes including p21, p27, and p57. However, although some investigators demonstrated that p21 decreased in regenerating rat liver (Timchenko et al., 1997), others reported its upregulation during cell cycle progression of rat hepatocytes in vivo (Albrecht et al., 1999; Jaumot et al., 1999; Kato et al., 1998). In addition, most reports described expression of CKIs in vivo during regeneration of rat or mouse liver. A lack of immunohistochemical analysis could not fully support the conclusion that observed patterns of CKIs expression in regenerating liver are fully derived from hepatocytes. Only a very limited number of reports documented patterns of p21 and p27 expression in hepatocytes during rat liver regeneration by immunocytochemistry (Jaumot et al., 1999; Pujol et al., 2000). Although a few reports described some aspects of CKIs expression in primary mouse and rat hepatocytes in pure culture (Albrecht et al., 1999; McIntyre et al., 1999), the functional role of these proteins in proliferating cells and their regulation by different signal transduction pathways have not yet been investigated .

2.2.4 Cyclins during liver regeneration

Cell cycle progression is regulated by sets of cyclin and cdk complexes. Cyclin D, -E, and -A are cyclically synthesized during G1, G1-S, and S phases, respectively. Cyclin D, with its catalytic partner Cdk4/6, leads to phosphorylation of Rb, releasing transcriptional factors such as E2F that activate genes required for entry into S-phase. (Fausto, 2000; Sherr, 1993). Concomitant binding of Cyclin E and A with their catalytic partner Cdk2 contributes to the initiation and progression of S-phase (Kitamura et al., 1998).

Quiescent cells contain low levels of D-type cyclins. Upon mitogenic stimulation, the expression of D-type cyclins increases and then Cyclin D-Cdk4 complexes can assemble and translocate to the nucleus (Michalopoulos and DeFrances, 1997; Fausto, 2000). The activation of Cyclin D-Cdk4 complexes at mid G1 is responsible for the phosphorylation of Rb and the other members of the pocket family (p107 and p130). As a result of this phosphorylation transcription factors of the E2F family are released and the expression of genes necessary for cell cycle progression is induced (Thompson et al., 1986; Alcorn et al., 1990; Morello et al., 1990; Feldenberg et al., 1999; Cressman et al., 1995). Cyclin D-Cdk4 activity is negatively regulated by CKIs, which consist of two major families. The Ink4 inhibitors (p15, p16, p18, and p19) specifically inhibit Cdk4 and Cdk6 by preventing complex formation with the D-type cyclins (Sherr and Roberts, 1995). The Cip/Kip family of proteins (p21, p27, and p57) bind and inhibit numerous cyclin-cdk complexes. The interplay between Cyclin D-Cdk4 and other G1-regulatory proteins is complex and incompletely understood . Cyclin D proteolysis requires phosphorylation by GSK3beta at Thr-286; additional work recently established that p286-D1 is a substrate for the SCF(Fbx4/alphaB-crystallin) E3 ligase (Barbash and Diehl, 2008).

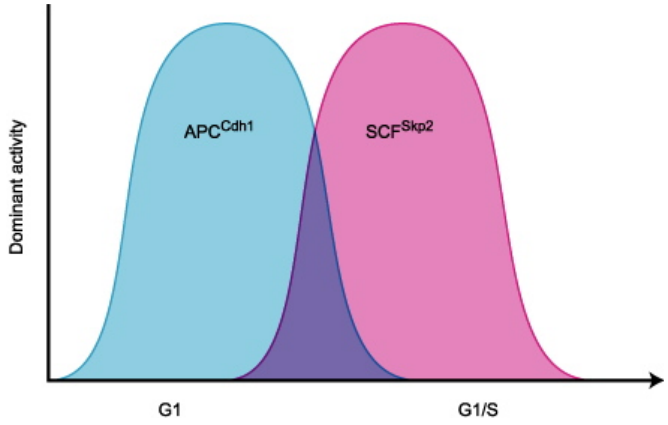

Figure 2.4:
Proteolytic ligase activity during G1 and early S phase. G1 stability is sustained by APC^{Cdh1} dependent degradation of G1 cyclins and SCF^{Skp2} dependent degradation of G1-S cyclins. The transition into S phase is mediated by a change in activity of the SCF^{Skp2} complex upon inhibition of APC^{Cdh1}. Thus, proteolytic targets are altered in a phase-dependent manner according to the selectivity of the active ubiquitin ligase at that time. Maintenance of subsequent phases and their transitions also involves similar mechanisms of phase specific selectivity of ligases (Ang and Harper, 2004).

Cyclin E-Cdk2 complexes formed at mid-late G1 also phosphorylate the pocket proteins. However, the major role of these complexes is accomplished in the G1-S transition, possibly by phosphorylating key proteins involved in the firing of DNA replication (Lundberg and Weinberg, 1998; Krude et al., 1997). Cyclin A-Cdk2 complexes are necessary for S phase progression although the putative substrates for these complexes are still unknown (Sherr, 1994).

2.3 Proteolytic degradation

Ubiquitin mediated proteolysis begins through an enzymatic cascade involving E1 ubiquitin activating enzymes, E2 ubiquitin conjugating enzymes, and E3 biquitin ligase enzymes. These enzymes ultimately serve to mediate the covalent attachment of Ub molecules by the E3 ligase to a lysine residue in the target substrate, or on the growing multi-ubiquitin chain extending from the "tagged" protein. This Ub chain serves as a signal for the 26S proteasome to unfold and digest the target. Most of the target selectivity of this system is conferred by the E3 ligase because of its direct interaction with the ubiquitination substrate. The two most prominent E3 ligases in cell cycle control are the anaphase-promoting complex (APC) and the SCF complexes [reviewed in (Hershko and Ciechanover, 1998)].

APC The APC core consists of 12 subunits and is regulated by two activating subunits: Cdc20 (also known as Fizzy) and Cdh1 (also known as Hct1 or Fizzy-related) (reviewed in (Vodermaier and Peters, 2004; Harper et al., 2002; Zachariae and Nasmyth, 1999)). These activating subunits

each confer differential substrate selectivity to APC. Moreover, each associates with the APC core under different circumstances: Cdc20 is more likely to associate with the APC core during mitosis under conditions of high Cdk activity, and Cdh1 is more likely to activate the APC core during mitotic exit and G1 phase under conditions of low Cdk activity. Substrate specificity of APC and its stage specific activation is discussed in more details in Chapter 3. Here we limit our discussion to Cdh1 associated APC and its role during G1 phase.

Cdh1 recognizes various proteins in late M and G1 phases, such as mitotic cyclins, Cdc20, Cdh1, Aurora A, Aurora B, Plk1, Skp2 (reviewed in (Castro et al., 2005)). Cdh1 levels are relatively constant throughout the cell cycle (Zachariae et al., 1998; Jaspersen et al., 1999). Cdh1 activity is regulated by cell cycle dependent phosphorylation and dephosphorylation, being unphosphorylated in late M and G1 phases, and then phosphorylated during S, G2 and early M phases (Zachariae et al., 1998; Jaspersen et al., 1999). The phosphorylation of Cdh1 by cyclin-cdk complexes inhibits APC activation by preventing Cdh1 from binding to the core APC subunits, whereas dephosphorylation of Cdh1 due to degradation of Cyclin B at late M phase and inactivation of Cdk1, induces APC activation by allowing Cdh1 to access the core APC subunits (Kramer et al., 2000). After Cdc20 is completely degraded at the end of mitosis, APC^{cdh1} remains as the active form of the APC during G1 and also during the first part of S-phase (Jaspersen et al., 1999; Kramer et al., 2000).

SCF The SCF E3 ligase comprises three subunits at its core; Skp1, Cullin, and Rbx1. This core interacts with modular F-box proteins, which all share an F-box sequence motif. F-box proteins directly bind to the target substrate and bridge the interaction between the E3 ligase and target substrate, and so the identity of the F box determines the target of SCF. There are various F-box proteins, including subfamilies with WD40 domains (Fbws) and those with leucine-rich-repeats (Fbls). F-box proteins frequently recognize their substrates through phosphorylation-dependent mechanisms. In the case of Fbws, the WD40 motif recognizes a phosphodegron domain on the substrate that forms after appropriate phosphorylation events [reviewed in (Deshaies, 1999; Koepp et al., 1999)].

The F-box-protein Skp2 bound to SCF complexes is most well studied among over 70 F-box proteins identified in human. SCF-Skp2 mainly ubiquitinates and degrades CKIs as well as G1-S cyclin (Frescas and Pagano, 2008). Controlling Skp2 activity is clearly important for proper cell cycle control. Skp2 can function as an oncogene in model systems and is overexpressed in a number of tumor types (reviewed in (Pagano and Benmaamar, 2003)). Bashir et al. (2004) and Wei et al. (2004) have indicated a Cdh1 dependent mechanism by which G1 cells maintain low levels of SCF^{Skp2} , thereby putting a break on S-phase entry until the criteria for S-phase entry have been met. Thus, G1 stability is maintained by APC^{Cdh1} dependent degradation of G1 cyclins and SCF^{Skp2} dependent degradation of G1-S cyclins. The transition into S phase is mediated by a change in activity of the SCF^{Skp2} complex upon inhibition of APC^{Cdh1}.

Therefore, proteolytic targets are altered in a phase-dependent manner according to the selec-

tivity of the active ligase at that time. Maintenance of subsequent phases of cell cycle and their transitions also involves similar mechanisms of phase specific selectivity of ligases(Fig. 2.4).

3 S phase and mitosis

Synopsis

Mitotic activation in mammalian cells is promoted by multiple redundant controls at the transcriptional, posttranslational and degradation level, but the relative contribution of these separate pathways to the decision to enter mitosis is currently not well resolved. There is a lot of emerging FoxM1 mediated transcriptional and Cdh1 mediated proteolytic control of Cyclin B in mammalian cells which is developing a new level of understanding for the mammalian cell cycle. This chapter summarizes the known molecular and cellular pathways observed in the control of mitotic cyclins and the mechanisms leading to activation, inactivation and degradation of cyclins.

3.1 Introduction

Early in the cell cycle, the DNA is replicated and chromosomes duplicated in the S phase. The second major phase of the cell cycle is the M phase, which typically consists of two events: nuclear division (mitosis) and cell division (cytokinesis). The period between the end of one M phase and the beginning of the next is called interphase.

Mitosis is a complex and beautiful process that distributes the duplicated chromosomes equally into a pair of daughter nuclei. The pairs of sister-chromatids are attached in early mitosis to the mitotic spindle, a bipolar array of protein polymers called microtubules. By the midpoint of mitosis (metaphase), sister-chromatids in each pair are attached to microtubules coming from opposite poles of the spindle. At the next stage (anaphase), sister-chromatid cohesion is destroyed resulting in sister-chromatid separation. The microtubules of the spindle pull the separated sisters to opposite ends of the cell (sister-chromatid segregation) and the two sets of chromosome are each packed into new daughter nuclei. Following mitosis cell itself divides by cytokinesis. Mitosis is preceded by gap phase called G2 when cells prepare themselves for mitosis (Fig. 3.1).

Cell cycle events are regulated at three regulatory checkpoints. First is the G1-S checkpoint which we have discussed earlier in chapter 2. There are two checkpoints during the G2/M transition where the progression of cell cycle can be blocked. Failure to complete DNA repli-

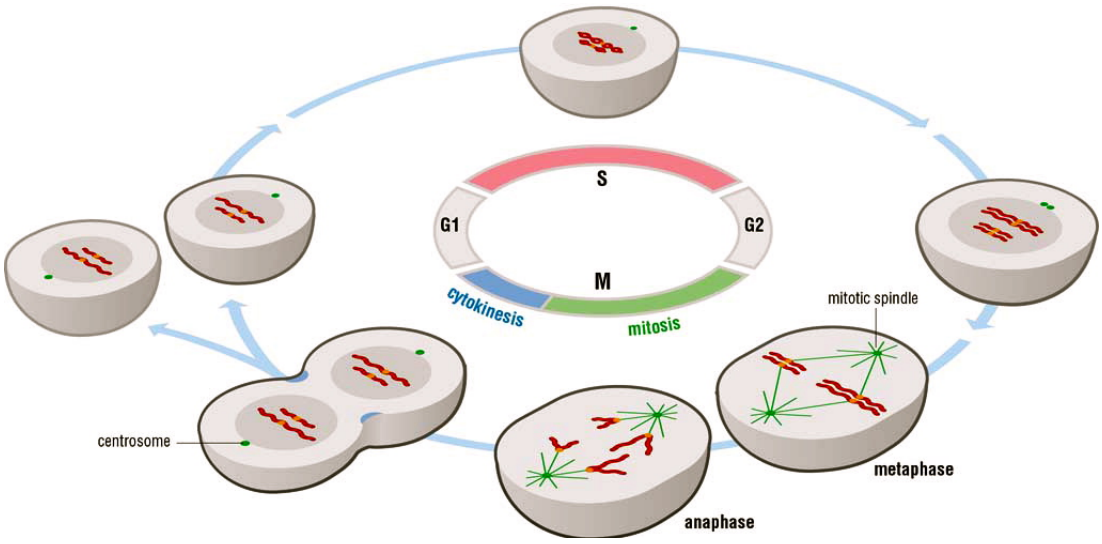

Figure 3.1:
Events of cell division cycle. The central events of cell reproduction are chromosome duplication, which takes place in S phase, followed by chromosome segregation and nuclear division (cytokinesis), which are collectively called M phase. G1 is the gap phase between S and M phases; G2 is the gap phase between S and M phases. At metaphase sister chromatids are aligned on the mitotic spindle and during sister chromatids get separated and pulled to opposite spindle poles (Morgan, 2007).

cation blocks the cell in G2 phase from entry into mitosis. Delay in spindle assembly blocks the metaphase-to-anaphase transition, thereby preventing sister-chromatid segregation until the spindle is ready. Cell cycle can thus be viewed as a linked series of tightly regulated molecular switches, each of which triggers the initiation of cell cycle progression at a specific regulatory checkpoint.

Cyclin oscillations regulating the cell cycle control system The whole process of cell division is mainly orchestrated by cdks. As the cells progress through the cell cycle, abrupt changes in the enzymatic activities of these kinases lead to changes in phosphorylation state and thus the state of activation of proteins that govern the cell cycle processes. Concentrations of cdks are constant throughout the cell cycle. Oscillations in their activity depend on the corresponding oscillations in the levels of their respective cyclin subunits. Different cyclins are produced at different cell cycle stages with additional controls imposed on them by various other cell cycle regulators (Fig. 3.3), resulting in a series of cyclin-cdk complexes which govern distinct cell cycle events (Fig. 3.2).

Cyclin D and Cyclin E form active complexes with cdks during G1-S transition which trigger DNA synthesis as discussed in chapter 1. The rise of G1-S cyclins is accompanied by the appearance of Cyclin A which also forms complexes with cdks. Cyclin A-Cdk2 is thought to initiate chromosome condensation during prophase. Towards the end of S phase, Cyclin B expression

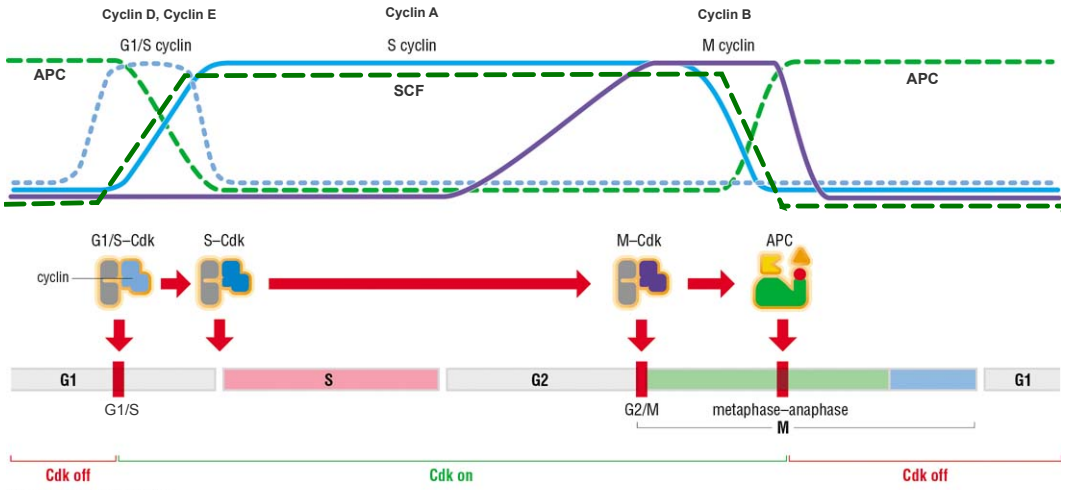

Figure 3.2:
Overview of the cell cycle control system. Levels of three major cyclin types oscillate during the cell cycle (top), providing the basis for oscillations in the cyclin-cdk complexes that drive cell cycle events (bottom). In general, cdk levels are constant and in large excess over cyclin levels. Thus, cyclin-cdk complexes form in parallel with cyclin levels. The enzymatic activities of cyclin-cdk complexes also tend to rise and fall in parallel with cyclin levels, although in some cases cdk inhibitor proteins or phosphorylation introduce a delay between the formation and activation of cyclin-cdk complexes. Formation of active G1-S cyclin-cdk complexes commits the cell to a new division cycle at G1-S checkpoint. G1-S cyclin-cdks then activate the S cyclin-cdk complexes that initiate DNA replication at the beginning of S phase. M-cdk cyclin activation occurs after the completion of S phase, resulting in progression through the G2/M checkpoint and assembly of the mitotic spindle. Proteolytic ligases (APC and SCF) impose another level of control on cell cycle oscillations. At the metaphase-to-anaphase transition APC activation triggers sister-chromatid separation. APC activation also causes the destruction of S and M cyclins and thus the inactivation of cdks, which promotes the completion of mitosis and cytokinesis. APC activity is maintained in G1 until G1-S cyclin-cdk activity rises again and commits the cell to the next cell cycle. G1-S cyclins are further degraded by SCF (adapted from Morgan (2007)).

is switched on, leading to accumulation of Cyclin B-Cdk1 complexes during G2. Switch like activation of Cyclin B-Cdk1 complexes trigger the G2-M transition (Fig. 3.4). Spindle assembly and other early mitotic events lead to the alignment of duplicated sister chromatids on the mitotic spindle in metaphase. In addition to that Cyclin B-Cdk1 eventually stimulates activation of APC, which triggers the metaphase-to-anaphase transition by stimulating the destruction of proteins that hold sister-chromatids together. The APC also causes destruction of S and M phase cyclins, resulting in the inactivation of all major cdk activities in late mitosis. Increased production of CKIs also occurs in late mitosis. The resulting inactivation of cdks allows dephosphorylation of their mitotic targets, which is required for spindle disassembly and the completion of M phase. Low levels of cdks are maintained until late in the following G1, when rising G1-S cyclins again commit the cell to a new cycle (Morgan, 2007).

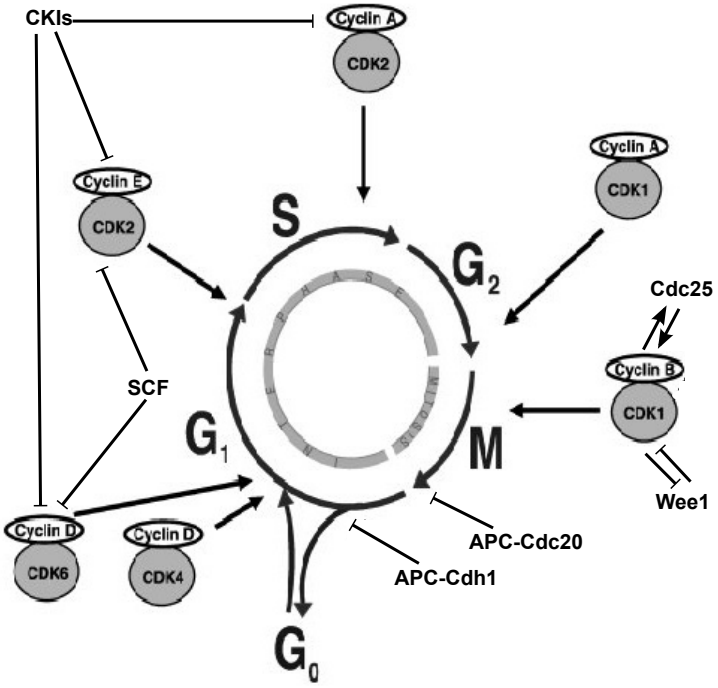

Figure 3.3:
Regulators of cell cycle control system. Cyclins are periodically expressed during cell cycle. Cyclin-cdk complexes are also formed in parallel with cyclins. Most crucial for the cell cycle transitions are the activity levels of cyclin-cdks. Properties like switch-like activation and delay in activation are introduced by various cell cycle regulators. CKIs prevent premature activation of Cyclin D, Cyclin E and Cyclin A by stoichiometrically inhibiting them. Cdc25 activates and Wee1 inactivates Cyclin B-Cdk1 in a positive feedback manner by phosphorylation-dephosphorylation events enabling switch-like activation of Cyclin B-Cdk1 at G2/M transition. Proteolytic degradators APC and SCF control the timely phase specific degradation of cyclins. APC with its activator subunit Cdc20 degrades Cyclin B at mitosis. With its another activator subunit Cdh1, APC degrades Cyclin B and Cyclin A till G1. SCF degrades Cyclin D and Cyclin E from Late G1 to S phase (adapted from Vermeulen et al. (2003)).

3.2 E2Fs regulating transcription of cyclins

E2F family of transcription factors are known to control the expression of various genes responsible for entry into and progression through S phase (e.g. Cyclin D, Cyclin E, Cyclin A). The applications of new technologies such as DNA microarray analysis, chromatin immunoprecipitation (ChIP) techniques and bioinformatics has enlarged the view of the number and nature of genes potentially regulated by E2F, including various G2 (e.g. Cyclin A) and M phase genes (e.g. Cdc2, Cyclin B) (Ren et al., 2002; Zhu et al., 2004; Osterloh et al., 2007).

E2F proteins can be (1) activators of transcription (E2F1, E2F2 and E2F3a) or (2) repressors of transcription (E2F3b, E2F4, E2F5 and E2F6) (Calzone et al., 2008a). For simplicity we only talk about the activator E2Fs and lump all three of them into one entity named E2F. E2F activity is tightly controlled by binding to retinoblastoma protein (Rb). Rb belongs to a family of pocket

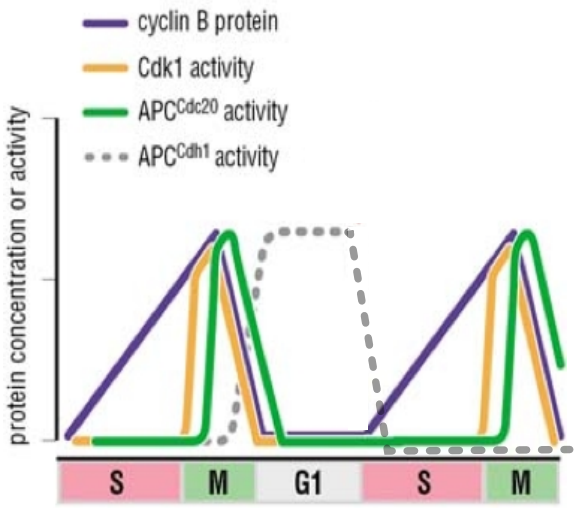

Figure 3.4:
Switch-like activation of mitotic players at G2/M transition. At G2/M transition the cell enters mitosis in an all-or-none fashion which is enabled by switch-like activation of Cdk1 associated with Cyclin B. Increased Cdk1 activity triggers the activation of APC^{Cdc20}, causing rapid Cyclin destruction and Cdk1 inactivation. Cdk1 inactivation, leads to APC^{Cdh1} activation, which further degrades Cyclin B till S phase (adapted from Morgan (2007)).

proteins which have the ability to bind proteins to their pockets. The protein binding function of Rb is regulated by phosphorylation. Rb sequesters E2F and inhibits its transcriptional activity. This hold of Rb on E2F depends on its phosphorylation level; higher the Rb phosphorylation level more the E2F is released from its hold and is available for transcriptional activation of further downstream genes.

Phosphorylation of Rb is regulated by various cdks. Complete phosphorylation and inactivation of Rb via cyclins occurs in a sequential and cooperative manner (Knudsen and Wang, 1996; Zarkowska and Mittnacht, 1997). At mid G1 Cyclin D-Cdk4/6 complexes initiate the phosphorylation of Rb. Cyclin D can only achieve partial phosphorylation of Rb. Complete phosphorylation of Rb and subsequent release of E2F in excess requires further phosphorylation of Cyclin D hypophosphorylated Rb by Cyclin E. Cyclin E hyperphosphorylates Rb during G1-S phase (Lundberg and Weinberg, 1998) (Fig. 3.5).

E2F together with B-myb is required for the activation of Cyclin B gene in G2. B-myb is an E2F target gene expressed at G1-S, but is not fully active until phosphorylated by Cyclin A-Cdk2 in S phase. This provides a possible explanation for delayed transcriptional activation of Cdc2 and Cyclin B in G2 (Zhu et al., 2004; Osterloh et al., 2007). Thus E2F also provides a link between G1-S and G2-M specific transcription and also provides a mechanism by which the temporal distinction is achieved. However, for simplicity reasons we do not explicitly consider B-myb mediated regulation of Cyclin B via Cyclin A.

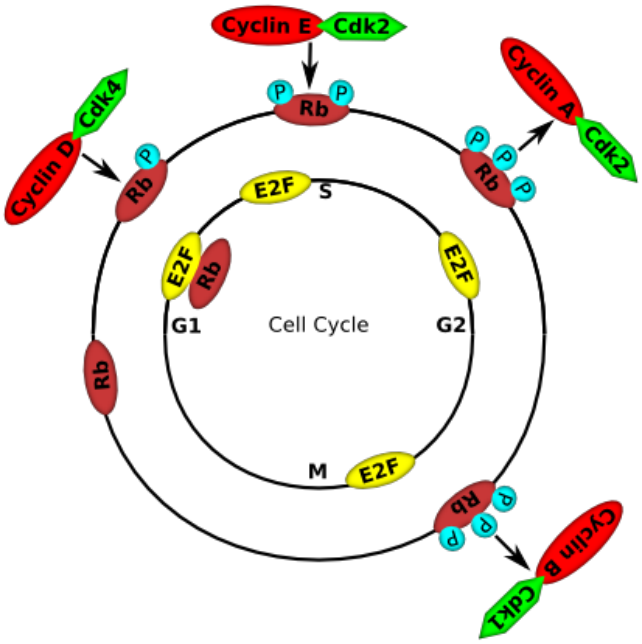

Figure 3.5:
Sequential activation of Rb-E2F. Phosphorylation of Rb is regulated by various cyclin-associated-kinases. Complete phosphorylation and inactivation of Rb via cyclins occurs in a sequential and cooperative manner. At mid G1, Cyclin D-Cdk4/6 complexes initiate the phosphorylation of Rb. Cyclin D can only achieve partial phosphorylation of Rb. Complete phosphorylation of Rb and release of E2F in excess requires phosphorylation of Cyclin D hypophosphorylated Rb by Cyclin E. Cyclin E hyperphosphorylates Rb during G1-S phase.

3.3 Cyclin dependent kinase inhibitors (CKIs)

Cyclin dependent kinase inhibitors (CKIs) provide additional regulation to the timely cell cycle dependent expression of cyclins. CKIs are grouped into two categories: Ink4 and Cip/Kip. Ink4 proteins inhibit Cyclin D associated kinase activity. Cip/Kip family consists of three members: p21, p27 and p57. These proteins share a homologous inhibitory domain, which is both necessary and sufficient for binding and inhibition of Cdk4- and Cdk2 containing complexes. These proteins act as stoichiometric inhibitors of Cdk2 and Cdk1; and they preferentially act on Cdk2 complexes (Vidal and Koff, 2000). p21 binds to all four cyclin-cdk complexes with a preference for those containing Cdk2 and inhibits their activation by generally blocking their catalytic sites. However, the mechanism behind CKIs dependent negative regulation of Cyclin B levels is still not clear (Gillis et al., 2009; Tyner, 2009).

p21 is predominantly transcriptionally regulated via IL-6 dependent STAT3, Myc and E2F (Gartel and Tyner, 1999; Coller et al., 2000), but recently its post-transcriptional control is reported to be equally important for its stability and degradation(Jascur et al., 2005; Sheaff et al., 2000). p27 mRNA levels remains majorly constant. Regulation of p27 protein is more complex involving

more posttranslational control (Pagano et al., 1995; Malek et al., 2001; Vervoorts and Luscher, 2008). p21 is also a transcriptional cofactor regulating the activity of E2F, STAT3, Myc and in turn, the transcription events regulated by them (Coqueret, 2003).

SCF^{Skp2} and APC^{Cdc20} promote the proteolysis of p21 bound to Cyclin E and -A in complex with Cdk2 or Cyclin A and Cyclin B in complex with Cdk1 (Wang et al., 2005; Amador et al., 2007).

All these observations suggest a complex two way role of CKIs in connecting S phase to mitosis via E2F, STAT3, Myc mediated transcriptional network and via proteolysis mediated by SCF and APC. Both ways directly or indirectly regulate activation of various cyclins.

3.4 Cyclins

Cyclin A Cyclin A has a function in both S phase and mitosis (Pagano et al., 1992) and it is associated with both Cdk1 and Cdk2 (Garnier et al., 2009). Cyclin A is known to be mainly an S-G2 phase cyclin starting to accumulate during S phase and is destroyed before metaphase. Transcription of Cyclin A is mediated by E2F transcription factor(Schulze et al., 1995).

Xenopus (Minshull et al., 1990), mice (Sweeney et al., 1996), and humans (Yang et al., 1997) contain two A type cyclins – Cyclin A1 and Cyclin A2. Cycin A is only expressed in meosis and very early embryos, whereas Cyclin A2 starts to accumulate during S phase and diminishes at early mitosis (Pines and Hunter, 1990). We limit our discussion to Cyclin A2 which is more S phase specific and we refer to it as Cyclin A for simplicity.

Cyclin A levels are low during G1, but Cyclin A increases at the onset of S phase, when it contributes to the stimulation of DNA synthesis (Resnitzky et al., 1995) both by initiating DNA replication and by restricting the initiation only once per cell cycle (Coverley et al., 2000; Petersen et al., 1999; Ishimi et al., 2000). The amount of Cyclin A remains high at S phase and in early mitosis. At early mitosis it associates with Cdk1 and stimulates entry into M phase (Draetta et al., 1989). Cyclin A in association with Cdk2 and Cdk1 also drives the initiation of chromosome condensation and possibly nuclear envelope breakdown (den Elzen and Pines, 2001; Furuno et al., 1999; Gong et al., 2007) . Hyperphosphorylated forms of Rb-E2F induce Cyclin A synthesis (Lundberg and Weinberg, 1998).

Timely disappearance of Cyclin A at the end of G2 phase is controlled by APC dependent porteolysis of Cyclin A (den Elzen and Pines, 2001). It is regulated by the D-box dependent recognition by APC proteosome like Cyclin B. Both APC^{Cdh1} and APC^{Cdc20} can promote ubiquitination of Cyclin A (Geley et al., 2001; Sudakin et al., 1995). Despite the similarity in degradation mechanisms of the two mitotic cyclins, there are clear differences in the destruction behavior of Cyclin A and Cyclin B considering its spindle-checkpoint independent destruction (Geley et al.,

2001).

APC^{Cdh1} degrades Cyclin A during G1 phase thus preventing its premature activation during G1 phase(Listovsky et al., 2000; Kramer et al., 2000; Listovsky et al., 2004; Rape and Kirschner, 2004). Premature activation of Cyclin A during late G1 phase is also controlled by its stoichiometric inhibition by p27 which forms an inhibitory complex with Cyclin A. p21 is also required to inhibit the M-phase-promoting activity of Cyclin A (Furuno et al., 1999).

Cyclin B Mitotic entry is catalyzed by the kinase activity of Cdk1 in complex with Cyclin B. Cyclin B-Cdk1 levels are periodically regulated by transcription and degradation cycles. Cyclin B levels first rise during G2, which allows the accumulation of Cyclin B-Cdk1 complexes. Phosphorylation-dephophorylation dependent activation of this Cyclin B-Cdk1 complex drives the cell cycle entry into mitosis; leading to enhanced chromosome condensation and nuclear envelope breakdown. Once all the chromosomes are attached to the mitotic spindle in bi-oriented fashion, progressive loss of Cyclin B-Cdk1 activity occurs. Cyclin B-Cdk1 inactivation and Cyclin B degradation is controlled by APC,leading to chromosome segregation and completion of cell division (Pines, 2006). Thus, there are three critical steps in the activation of Cyclin B-Cdk1 complex: 1) Transcriptional levels of Cyclin B available to form a complex with Cdk1 (Solomon et al., 1990). 2) Phosphorylation level of Cdk1 which is regulated by the opposing activities of Wee1 and Cdc25 (O'Farrell, 2001). 3) Degradation of Cyclin via APC proteasomal subunit (Fig. 3.6).

Transcription of Cyclin B starts in S phase and peaks in late G2. Several transcription factors including E2F, NF-Y, FoxM1, and B-Myb have been shown to activate transcription of Cyclin B promotor (Lindqvist et al., 2005). All these transcription factors are activated by cdk activity, ensuring that transcription of Cyclin B is efficient only when Cyclin A-Cdk2 activity builds up during S and G2 phase (Chae et al., 2004; Osterloh et al., 2007; Laoukili et al., 2008). When the levels of Cyclin B reach a threshold concentration Cyclin B-Cdk1 starts being activated (Solomon et al., 1990).

Thus, precise temporal regulation of Cyclin B-Cdk1 activity is ensured by the coordinated action of various positive and negative regulators of Cdk1 activity which form various feedback loops around Cyclin B-Cdk1.

3.5 Proteolytic degradation

Degradation of Cyclin B is regulated by APC, a multisubunit E3 ligase that can only polyubiquitinylate many mitotic targets regulators to target them for destruction by the proteasome. Polyubiquitinylation of Cyclin B starts in metaphase, when the spindle assembly checkpoint is silenced (Acquaviva and Pines, 2006; van Leuken et al., 2008). APC continues to promote

degradation of Cyclin B until early S phase (Lukas et al., 1999; Hsu et al., 2002).

Ubiquitin-mediated protein degradation plays a key role in the regulation of cell cycle. Attachment of a polyubiquitin chain to a protein targets that protein for degradation by the 26S proteasome (Coux et al., 1996; Baumeister et al., 1998). Ubiquitin becomes covalently attached to a substrate by interaction with a cascade of ubiquitin protein ligase enzymes (E1, E2 and E3) in a three step process. The last in the series, E3, transfers the ubiquitin to the substrate protein (Hershko and Ciechanover, 1998).

Two ubiquitin protein ligases play a role in the cell cycle: the SCF (Skp1/Cullin/F-box) complex and the APC (anaphase-promoting complex) (Peters, 1998; Zachariae and Nasmyth, 1999). Both are multi-subunit complexes that recognize the substrate protein and bring them face-to-face with the proteosome. Both complexes are known as the proteolytic machinery of the cell cycle degrading the cell cycle proteins. Recent studies have established their role much beyond degradation of cyclins. APC-type ligases regulate entry into and exit from mitosis, whereas SCF^{Skp2} controls entry into S phase. APC^{Cdc20} degrades the G2-M substrates while APC^{Cdh1} degrades the late mitotic and G1 substrates. By targeting the SCF-component Skp2 for destruction, APC^{Cdh1} not only regulates exit from mitosis, but also controls the duration of G1 (Kurland and Tansey, 2004) (Fig. 3.7).

SCF The SCF complex consists of three invariable components: Rbx1 (Ring-finger protein), Cul1 (scaffold protein), and Skp1 (adaptor protein), as well as one variable component called F box protein that binds to Skp1 and is responsible for substrate recognition. Three F box proteins : Skp2 (S-phase kinase-associated protein, Fbw7 (F-box and WD-40 domain protein 7 (Fbw7) and β-TRCP (β-transducine repeat-containing protein), are thought to be involved in cell cycle control (Nakayama and Nakayama, 2006) (Fig. 3.8).

SCF although originally thought to function mainly at the G1-S transition, is active from late G1 to early M phase (Nakayama and Nakayama, 2006). In conjunction with the F box protein Skp2 , the SCF complex targets CKIS ((Carrano et al., 1999), (Bornstein et al., 2003), (Kamura et al., 2003)) for degradation; which are the breaks that antagonize the cell cycle progression by inhibiting various cyclins. Skp2 is also reported to degrade many accelerators of cell cycle progression like Cyclin D (Yu et al., 1998; Lin et al., 2006), and Cyclin E (Nakayama et al., 2000) (Fig. 3.9). Cyclin E is targeted for proteasomal degradation also by another F box protein subunit of SCF, Fbw7 (Koepp et al., 2001; Strohmaier et al., 2001) (Fig. 3.8) (Fbw7 subunit of SCF not depicted in the figure for simplicity). Thus, SCF helps in removing S phase blocks by degrading cell cycle breaks imposed by CKIs and allows DNA replication by degrading accelerators of cell cycle progression.

APC The APC ubiquitination complex is structurally similar to the SCF complex, and consists of invariable core components : APC11 (Rbx1-related RING-finger protein), APC2 (CUL1-

related scaffold protein) and at least 11 other components without a defined role, as well as variable components known as activator. There are two such variables in mitotically cycling cells: cell division cycle 20 (Cdc20) and Cdh1 (Fig. 3.8). They confer substrate specificity in the same way that F box proteins do in the SCF complex (Harper et al., 2002; Castro et al., 2005). The APC is active from mid-mitosis (anaphase) to the end of G1 phase (Nakayama and Nakayama, 2006). Cdc20 and Cdh1 recognize proteins that have a destruction box (D box) or a KEN box (Pfleger and Kirschner, 2000; Burton and Solomon, 2001). The windows of activity of APC^{Cdc20} and APC^{cdh1} are clearly different: the former is active from mid-mitosis (anaphase) to late mitosis, whereas the later is activated at late mitosis, remains active through G1 phase and is extinguished at the G1-S boundary (Harper et al., 2002; Castro et al., 2005) (Fig. 3.10).

APC^{Cdc20} contributes to the proteolysis of securin, and thereby triggers chromosomal separation at anaphase. After replication, sister chromatids are accompanied until anaphase by the multi-protein complex cohesin. Separase cleaves cohesin, but its activity is suppressed by securin. The degradation of securin by APC^{Cdc20} activates separase, resulting in the cleavage of cohesin and separation of sister chromatids (Uhlmann et al., 1999, 2000; Yanagida, 2000) (Fig. 3.10). Although Cdc20 is expressed in G2 phase before the entry to M phase, the activity of APC^{Cdc20} is suppressed until spindle attachment at kinetochores is completed in mitosis. This surveillance system, known as the spindle checkpoint, precludes precocious segregation of chromosomes, which would result in abnormal chromosome number (aneuploidy), a prevalent form of genetic instability in human cancers. The main mediators of this system are mitotic arrest deficient (Mad) and budding uninhibited by benzimidazole (Bub) proteins which bind to, and inhibit, the function of Cdc20 (Bharadwaj and Yu, 2004). Once all kinetochores are attached to spindles at the metaphase-anaphase transition the MCC is dissociated from CDC20, resulting in the activation of APC^{Cdc20} and chromosome segregation (Harper et al., 2002; Castro et al., 2005) (Fig. 3.10).

APC^{Cdh1} on the other hand, recognizes broad range of proteins from late M and G1 phases, such as mitotic cyclins, Cdc20, Cdh1, Aurora A, Aurora B, Plk1, Skp2 (reviewed in (Castro et al., 2005)). Unlike Cdc20, Cdh1 levels are relatively constant throughout the cell cycle (Zachariae et al., 1998; Jaspersen et al., 1999). Cdh1 activity is regulated by cell cycle dependent phosphorylation and dephosphorylation, being unphosphorylated in late M and G1 phases, and then phosphorylated during S, G2 and early M phases (Zachariae et al., 1998; Jaspersen et al., 1999). The phosphorylation of Cdh1 by cyclin-cdk complexes inhibits APC activation by preventing Cdh1 from binding to the core APC subunits, whereas dephosphorylation of Cdh1 due to degradation of M phase cyclin, Cyclin B and inactivation of Cdk1, induces APC activation by allowing Cdh1 to access the core APC subunits (Kramer et al., 2000). After Cdc20 is completely degraded at the end of mitosis, APC^{cdh1} remains as the active form of the APC during G1 and also during the first part of S phase (Jaspersen et al., 1999; Kramer et al., 2000) (Fig. 3.10). APC^{cdh1}, degrades Cyclin A and -B till G1 (Kramer et al., 2000; Listovsky et al., 2004; Rape and Kirschner, 2004). Rise in Cyclin A levels during late G1 and S phosphorylates Cdh1 and prevents its activatory assembly with APC (Lukas et al., 1999)

Bashir et al. (2004) and Wei et al. (2004) independently demonstrate that APC^{Cdh1} target the subunits of SCF, *viz*, Skp2 and Cks1 for ubiquitination and destruction during G. SCF being degradator of CKIs is thus consequently prevented APC^{Cdh1} from mediating premature degradation of CKIs in G1. This in turn prevents premature S phase. These studies reveal a circuit that can stabilize the G1 state through the down-regulation of S-phase promoting factors by an indirect proteolytic mechanism dependent on APC^{Cdh1}.

3.6 Additional feedback controls

Sheer association of Cyclin B with Cdk1 is not sufficient for its full activation, as the Cdk1 subunit undergoes posttranslational modifications via various phosphorylation and dephosphorylation events that affect its kinase activity. During interphase CyclinB-Cdk1 complexes are kept inactive by phosphorylation of Cdk1 at specific sites by Wee1 kinases. At G2/M transition Wee1 is inactivated while the dual specificity phosphatase, Cdc25, is activated. Cdc25 dephosphorylates Cdk1, allowing its activation and entry into mitosis.

Just as Cyclin B-Cdk1 activation is highly regulated, both Wee1 and Cdc25 are tightly controlled through the cell cycle. Cdc25 and Wee1 are similarly regulated, though their activities oscillate in opposition to one another, consistent with their role of inhibiting or activating mitotic entry. Both are directly controlled by mitotic kinases like Cyclin B-Cdk1. Wee1 keeps Cyclin B-Cdk1 inactive by phosphorylation, while Cdc25 removes this inhibitory phosphorylation.

Cdc25 proteins are dual specificity phosphatases that uniquely function to dephosphorylate specific tyrosine/threonine residues on cdks. Three isoforms of Cdc25 are known in mammalian cells: Cdc25A, -B, -C which cooperate to activate Cyclin B-Cdk1 complexes during the G2M transition (Lindqvist et al., 2005; Boutros et al., 2006). All three isoforms of Cdc25 are responsible for dephosphorylating Cdks on Thr14 and/or Tyr15 residues. This dephosphorylation triggers the final activation of Cylin B-Cdk1 during G2/M transition. Complete activation of Cyclin B-Cdk1 requires phosphorylation by Cdc25C at the onset of mitosis (Boutros et al., 2006).

The levels of Cdc25 proteins expressed at each stage of the cell cycle depends on the balance between protein synthesis and degradation. While Cdc25C protein levels remain fairly constant throughout normal cell division (Girard et al., 1992), those of Cdc25 A and -B vary in a cell cycle dependent manner (Boutros et al., 2006). Cdc25A is expressed from mid to late G1 (Boutros et al., 2006) and is degraded by ubiquitin-mediated proteolysis at the end of mitosis (Donzelli et al., 2002). Cdc25B accumulates from S phase and peaks in mitosis (Gabrielli et al., 1996; Lindqvist et al., 2004) and is degraded by ubiquitin mediated proteolysis following phosphorylation by Cyclin A-Cdk1 (Baldin et al., 1997).

Three isoforms of Cdc25 are lumped together into a single entity for simplicity and named as Cdc25 in future discussions, unless stated otherwise. Cdc25 activity and phosphorylation

levels are kept low during interphase in the presence of incompletely replicated DNA (Izumi et al., 1992; Kumagai and Dunphy, 1992). Cyclin A and Cyclin B mediated phosphorylation of Cdc25 renders it active (Izumi and Maller, 1993). Thus mitotic cyclins activate Cdc25 by phosphorylation which further activates CyclinB-Cdk1 in a positive feedback loop manner.

Wee1 constitutes another positive feedback to Cyclin B-Cdk1 in the form of a double negative regulation. Cyclin B-Cdk1 activity can also be positively regulated by negative regulation of its inactivators Wee1 and Myt1 by phosphorylation on two inhibitory residues - T14 and Y15. Wee1 is a tyrosine kinase that phosphorylates Y15 (Parker and Piwnica-Worms, 1992), and Myt1 is a dual-specificity kinase that can phosphorylate both sites (Mueller et al., 1995). During G2/M transition Wee1 and Myt1 kinases are phosphorylated and inactive when Cyclin B-Cdk1 activity rises. After mitotic exit, Wee1 and Myt1 kinases become activated and can lock Cdk1 in the inactive state, thus serving to inhibit Cdk1 during G1 (Potapova et al., 2009). Wee1 is positively regulated during interphase by autophosphorylation (Murakami et al., 1999).

These two Wee1 and Cdc25 mediated phosphorylation and dephosphorylation mechanisms serve as positive feedback that induces a robust autoamplification of Cdk1 activity (O'Farrell, 2001), and they are responsible for the switch like activation of Cdk1(Pomerening et al., 2005).

Spatial regulation of cyclin B concentration in the nucleus and cytoplasm also adds up another level of complexity in its regulation. For simplicity spatial control of Cyclin B is not considered for the modeling.

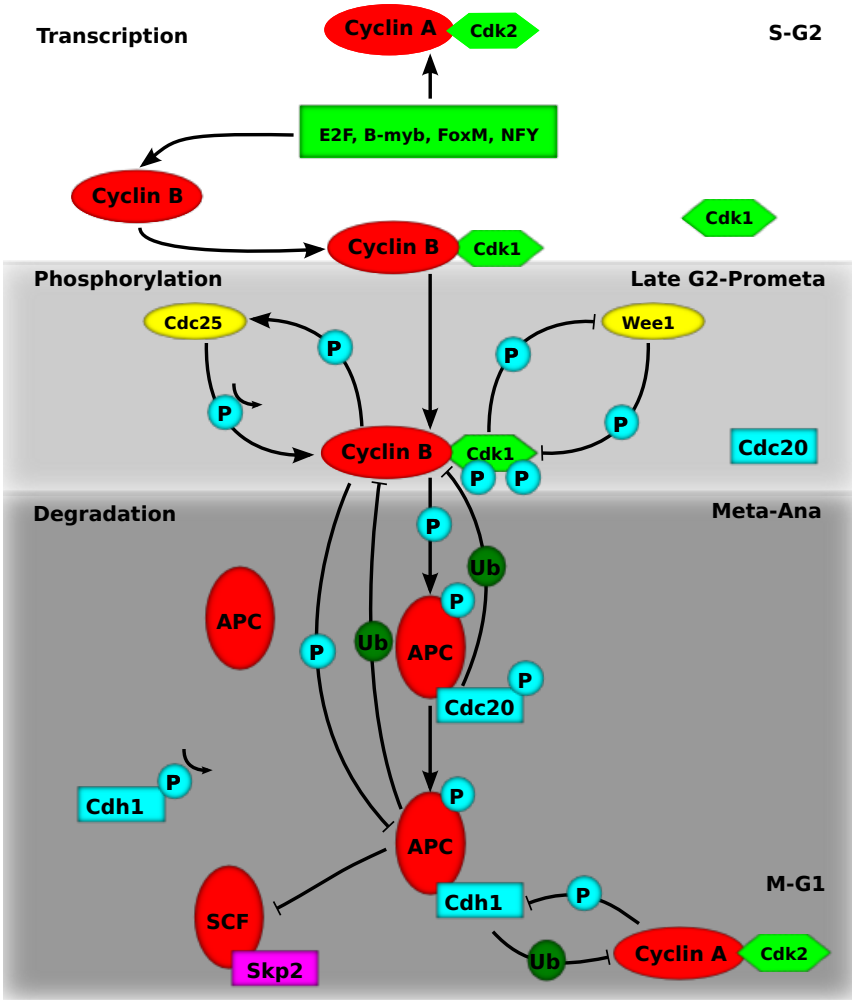

Figure 3.6:
Cyclin B regulation. Cyclin A and Cyclin B activity build up during S and G2 phase due to increased transcription of mitotic genes such as E2F, NF-Y, FoxM1, and B-Myb. At G2-M, mutually antagonistic inactivation of Wee1 by Cyclin B-Cdk1 and activation of Cdc25 by Cyclin B-Cdk1 impose a double positive feedback on Cyclin B activity. Thus, leading to abrupt full activation of Cyclin B-Cdk1 resulting in a switch-like G2/M transition. Degradation of Cyclin B is carried out by APC Ubiquitin-ligases, when the spindle assembly checkpoint is silenced. At metaphase Cyclin B-Cdk1 activates its degradator APC^{Cdc20}, while during late mitosis degradation of Cyclin B leads to the activation of APC^{Cdh1} which continues degradation of cyclins till late G1 phase. At G1 phase APC^{Cdh1} also degrades SCF which is a degradator of G1-S phase cyclins, thus connecting mitosis to G1 phase. Therefore, precise temporal regulation of Cyclin B-Cdk1 activity is ensured by the coordinate action of various positive and negative regulators of Cdk1 activity and degradators of cyclins which form feedback loops around Cyclin B-Cdk1.

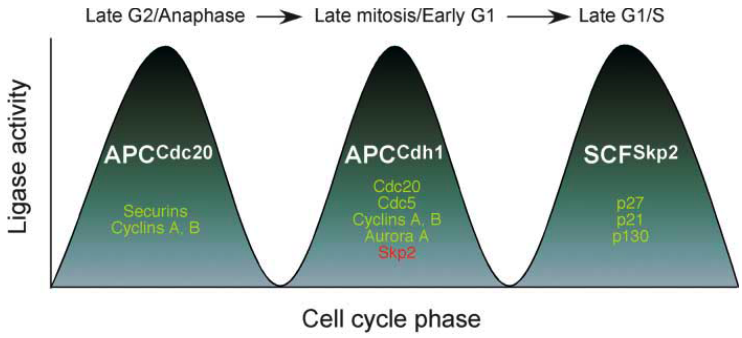

Figure 3.7:
Cell cycle driven by waves of Ub ligase-dependent protein degradation. By destroying specific substrates, APC-type ligases regulate entry into and exit from mitosis, whereas SCF^{Skp2} controls entry into S phase. APC^{Cdc20} degrades the G2-M substrates while APC^{Cdh1} degrades the late mitotic and G1 substrates. By targeting the SCF-component Skp2 for destruction, APC^{Cdh1} not only regulates exit from mitosis, but also controls the duration of G1 (Kurland and Tansey, 2004). Because of the central role of Cdh1 in controlling mitotic and G1 substrates, loss of Cdh1 has implications in exit from quiescence, G2 delay and mitotic aberrations.

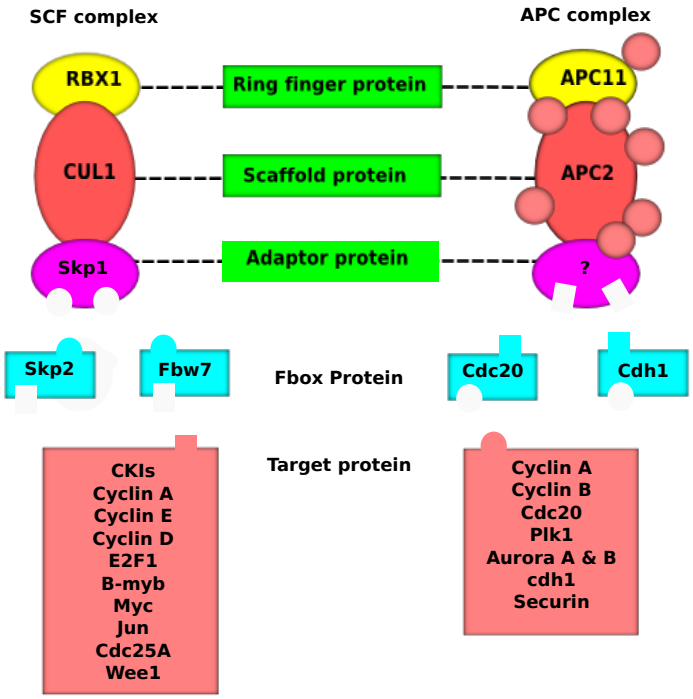

Figure 3.8:
APC and SCF structure. The SCF complexes consist of three invariable components: Ring-finger protein (Rbx1 in SCF, APC11 in APC), scaffold protein (Cul1 in SCF, APC2 in APC), and adaptor protein (Skp1 in SCF and, not yet well characterized in APC), as well as one variable component called F box protein that binds to adaptor protein and is responsible for substrate recognition. SCF related F box proteins such as Skp2 and Fbw7 recognize G1-S substrates mainly. Among APC related F box proteins, Cdc20 recognizes mitotic substrates, whereas Cdh1 is less specific and can also recognize a broad range of substrates including Cyclin A, Cyclin B and Skp2 (adapted from Nakayama and Nakayama (2006)).

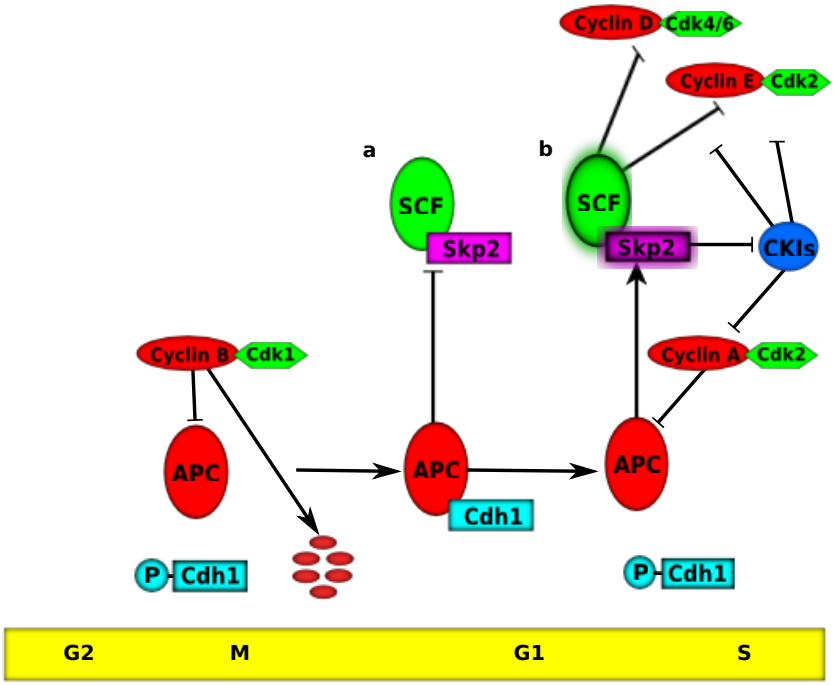

Figure 3.9:
Regulation and function of SCF. The SCF is active from late G1 to early M phase (Nakayama and Nakayama, 2006). In conjunction with Skp2 , the SCF targets CKIs for degradation. CKIs are inhibitors of G1-S phase cyclins (Cyclin D, Cyclin E and Cyclin A). Thus, SCF contributes in removal of S phase blocks by degrading cyclin inhibitors, CKIs, and allows DNA replication by degrading G1 and S phase cyclins (adapted from Nakayama and Nakayama (2006)).

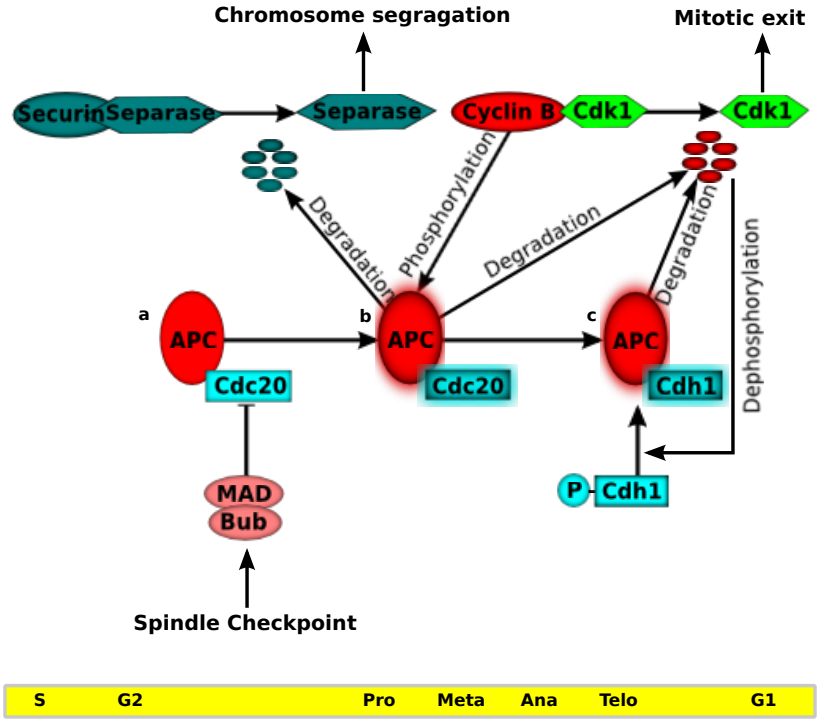

Figure 3.10:
Regulation and function of APC. (a) APC^{Cdc20} is kept inactive at G2 by spindle checkpoint proteins Bub and Mad. (b) At metaphase, Cyclin B-Cdk1 phosphorylates and activates APC^{Cdc20} which then degrades securin freeing separase to assist in cleaving sister chromatids, thus leading to chromosomal segregation. (c) Further, APC^{Cdc20} degrades Cyclin B, leading to mitotic exit. Degradation of Cyclin B also results in dephosphorylation of Cdh1, which then attaches to APC to form active APC^{Cdh1} complex. APC^{Cdh1} then continues the job of degrading mitotic and G1 cyclins (adapted from Nakayama and Nakayama (2006)).

4 Cell cycle modeling: state of the art

4.1 Introduction

Study of the cell cycle began with the discovery of cell division. In 1960s first mathematical models of cell cycle came into existence that tried to explain some key aspects of cell cycle regulation from phenomenological observations (Koch and Schaechter, 1962; Shields, 1977; Smith and Martin, 1973). These early efforts regarded the machinery that controlled the cell division cycle as a black box, and attempted to produce a mathematical description of the rules it obeyed. The landscape changed dramatically in the 1980s, as work on yeasts and the rapid cell divisions of fertilized eggs produced a molecular description of the cell cycle oscillator as a fluctuation in the activity of Cdks that is driven by a combination of phosphorylation reactions and the periodic destruction of the cyclins that activate Cdks (Norel and Agur, 1991; Goldbeter, 1991; Thron, 1991; Tyson, 1991) when some data on the underlying molecular regulatory network came to light (Nurse, 1990). Experimentally, this oscillation can depend on a single Cdk – Cdk1, also known as Cdc2 or, in budding yeast, as Cdc28 – and a single cyclin, cyclin B. These discoveries quickly inspired a new type of mathematical analysis, one which attempted to represent the molecular details of the cell cycle oscillator and the pathways that it interacted with as differential equations.

In recent years, with the birth of systems biology (Kitano, 2002), new experimental techniques have led to an extension of these models, and there now appears to be a bright future for models of cell cycle regulation. Several excellent reviews are available on computational modeling techniques (Gilbert et al., 2006; Karlebach and Shamir, 2008), on cell cycle regulation (Morgan, 2007; Nurse, 2000; Tyers, 2004)and on cell cycle modeling (Csikasz-Nagy et al., 2008b; Fuss et al., 2005; Ingolia and Murray, 2004; Sible and Tyson, 2007; Tyson and Novak, 2008).

4.2 Cell cycle differences between different model organisms

After it took a century to confirm that cell replication is a controlled process, the last three decades were spent to cast the current cell cycle regulation model. The essential achievement in identifying the key components and in dissecting the mechanisms of the cell cycle circuitry has been attributed to the simultaneous use of model systems like yeast, frogs, sea urchin, starfish,

and flies. Now is the time to investigate whether those findings also apply to mammalian in vivo models like mice and eventually can be used for effective disease therapy in humans.

The intrinsic biological advantages of model organisms have facilitated the understanding of the cell cycle. For instance, the mitotic cell cycle of frog oocytes and early embryos proceed without the G1 and G2 phases because maternally inherited materials govern the progression of cell division. Therefore frog is an ideal model for dissecting DNA replication and mitosis.

Similarly, Drosophila only expresses a single D-type as well as E-type cyclin that could ease the analysis of the G1 and S phases. However, the potential drawback of this simplicity is that the model might not reflect the cell cycle regulation that occurs in humans and may therefore hinder the interpretation and translation for clinical purposes.

In contrast, yeasts have a typical G1-S-G2-M cell cycle pattern and a short cell cycle of around 2 h. In addition, yeasts have multiple cyclins allocated to a single cyclin dependent kinase (Cdk1) resembling the requirement of mammals. These characteristics of yeasts made them potent candidates for mutagenesis and genetic studies of the cell cycle regulation (Hartwell et al., 1970; Nurse et al., 1976). One caveat of the yeast model is that it is based on only one cyclin-dependent kinase, Cdk1, interacting with multiple oscillating cyclins (Cln1/2/3 and Clb1/2/3/4/5/6) during different phases of the cell cycle. In addition, Cdk1/cyclin B (Cdc2/Cdc13) complexes alone are sufficient to drive cells to complete the S phase and mitosis (Fisher and Nurse, 1996). This situation does not apply to the mammalian cell cycle where multiple Cdks and cyclins interact to promote cell cycle progression and development as described below. Therefore, the genetic redundancy of the activating cyclins, the inhibiting as well as the promoting factors in yeast may not exactly reflect the situation in mammals. Despite the intrinsic disadvantages of these aforementioned models, they do provide broad references for cell cycle studies in the mouse, and by analogy in humans.

4.3 Cell cycle models

As mentioned above the story goes back about 50 years, when Prescott found that cells need to reach a critical size to divide [95]. This and other phenomenological observations drove the first wave of mathematical models that tried to understand how cell progresses through its different phases to finally divide into two daughter nuclei. Once the molecular interactions that control the cell cycle were discovered many groups started to work on mathematical models to figure out the key concepts of these interactions.

Yeast cell cycle models

The most studied organism in the modeling platform is budding yeast *Saccharomyces cerevisiae*. A detailed model of cell cycle regulation in *Saccharomyces cerevisiae* is developed by Novák and Tyson group. They proposed that a different hysteretic switch controlled the entry into S phase (Chen et al., 2000) which was first tested experimentally by Fred Cross's group in a seminal paper focusing solely on verifying a mathematical model of cell cycle regulation (Cross et al., 2002). Later they created a model that can simulate the behavior of more than 120 mutants (Chen et al., 2004). This model also predicted the existence and regulation of a phosphatase that later was identified (Queralt et al., 2006). Other groups have also presented their own models of the budding yeast cell cycle, focusing on various aspects of the regulatory system (Braunewell and Bornholdt, 2007; Barberis et al., 2007; Sriram et al., 2007; Stelling and Gilles, 2004).

The other favorite test organism of cell cycle research is the fission yeast Schizosaccharomyces pombe, for which there exist models describing its DNA replication (Lygeros et al., 2008; Novak and Tyson, 1997), cell division (Csikasz-Nagy et al., 2007) and the behavior of some interesting mutants (Sveiczer et al., 2000; Steuer, 2004).

Embryonic cell cycle models

Embryonic cell cycles have been modeled not only in frogs but also in the fly, Drosophila melanogaster (Calzone et al., 2007), and in the sea urchin (Ciliberto and Tyson, 2000).

The group of Béla Novák and John J. Tyson investigated the regulation of mitotic entry in eggs of the frog *Xenopus laevis* and found that a model with two positive feedback loops could provide a reliable switch for entry into mitosis (Novak and Tyson, 1993). Their model predicted that the Cdk control system can be bistable: under certain conditions, Cdk may be either active or inactive depending on the recent history of the cell. This bistability and hysteresis was verified experimentally 10 years later in a seminal paper by Pomerening et al. (Pomerening et al., 2003; Sha et al., 2003).

Mammalian cell cycle models

The most challenging task is to model cell cycle regulation in mammalian cells, where multiple control mechanisms exist that hold cells back from proliferation. The physiological differences among different types of mammalian cells make this task especially difficult. Cancer cell lines are often (possibly always) perturbed in their cell cycle regulation (Hanahan and Weinberg, 2000), thus most existing models describe 'generic' proliferating mammalian cells at various levels of detail. A few of these models use some data on mouse fibroblasts; still, no model of the

cell cycle network of a specific mammalian cell type has yet been constructed. Several models exist that do not focus on any specific cell type but rather investigate some important aspects of the regulatory modules of the general Cdk control network. These approaches are biologically suitable, since it has been shown that the key cell cycle controllers and their interactions are universal among eukaryotes (Nurse, 1990). Also, naturally synchronized cells in the whole animal systems, such as hepatocytes in regenerating livers of mice, are being used to analyse the cell cycle of naturally synchronized cells in the whole animal (Chauhan et al., 2008)

Prokaryotic cell cycle models

Recent modeling studies on cell cycle regulation of the prokaryote Caulobacter crescentus show that, even though the key controller genes are completely unrelated to their eukaryotic counterparts, the network wiring resembles the eukaryotic system (Brazhnik and Tyson, 2006; Li et al., 2008). This conservation of network structure underlines certain key features of cell cycle regulation.

4.4 Modeling methodologies

Positive and negative feedback loops have to be wired together for proper cell cycle regulation. The positive feedbacks are important for robust transitions between cell cycle phases and they assure that checkpoints can stop progression through the cell cycle, while the negative feedbacks are necessary to reset the system to the beginning and drive the periodic repetition of the process (Novak et al., 2007). The significance of positive feedback in robustness of cell cycle transitions has recently been shown in different organisms (Holt et al., 2008; Pomerening et al., 2005; Skotheim et al., 2008; Legewie et al., 2006, 2005).

Most of the above mentioned models based on molecular networks use systems of ordinary differential equations (ODEs) to describe the dynamics of the system. This allows the use of some mathematical analysis tools that can track the steady states and dynamical transitions of cell cycle control system (Battogtokh and Tyson, 2004; Borisuk and Tyson, 1998; Swat et al., 2004; Csikasz-Nagy et al., 2006). As the complexity of the known cell cycle regulatory network increased in the last few years, logical dynamic modeling (Thieffry, 2007) and especially Boolean algebra became another fashionable modeling formalism . This might be partially influenced by the success of Li et al. (Li et al., 2004), who showed in a logical model of the budding yeast cell cycle that trajectories from 86 % of all possible initial states lead the system into one state representing G1-phase of the cell cycle. Most of these trajectories funneled into a path which steps through the different phases of the cell cycle, showing that the cell cycle is robustly designed.

Although some of these logical models were already using stochastic updating, recently some

much more detailed formalisms have started to consider the effects of molecular noise in the cell cycle regulatory network (Gillespie, 2007). These stochastic models can investigate how individual cells might differ from the average behavior of the population (the output of deterministic ODE models). Stochastic fluctuations could be relevant for certain mutant cell populations that show partial viability (Mura and Csikasz-Nagy, 2008). Furthermore, recent advances in experimental observations on single cells allow us to measure the distribution of behaviors in a population of cells, for example, the measurements of the noisiness of the G1/S transition in budding yeast cells provided by Cross's group (Bean et al., 2006; Di Talia et al., 2007).

4.5 Advances and challenges in cell cycle modeling

Need for comprehensive databases

Comprehensive databases force modelers to face new challenges. They have to handle somehow this huge amount of data, develop platforms to build large models, and find the suitable methods to analyze them. Conventional, hand-written systems of ODEs have been studied by numerical simulations, sensitivity analysis and bifurcation theory, in order to understand the model's behavior. As our knowledge base is growing, we have reached a point where we need new tools to build large models (Kitano et al., 2005), to code them in a platform-free language (Hucka et al., 2003) and to store them for community use (Le Novere et al., 2006; Olivier and Snoep, 2004). For example, cell cycle models now have their own database with links to experimental data (Alfieri et al., 2007).

Parameter optimization

Several modeling platforms have been used in cell cycle research (Dematté et al., 2008; Ermentrout, 2002; Schmidt and Jirstrand, 2006; Vass et al., 2004). These usually guide the user from model building to some type of analysis. JigCell has been developed precisely for cell cycle model simulations and data fitting (Vass et al., 2004). It can run multiple parameter sets to simulate various mutants and it includes a comparator that can test how well the simulations fit physiological details of mutants. Although it is difficult to define a suitable objective function for data that is not time dependent, JigCell provides tools for such estimations (Panning et al., 2008). Indeed parameter optimization is one of the major challenges for modeling. High-throughput measurements rarely give reliable kinetic rates; most often they should be estimated from concentration profiles by a parameter optimization algorithm (Hoops et al., 2006; Lecca et al., 2009; Saez-Rodriguez et al., 2008; Zi and Klipp, 2006; Zwolak et al., 2005).

Missing interactions in the biological network

Search for missing rate values is just one part of the job that computational tools can do for us. All models we create are some abstractions of the real biological system, thus we know that we are missing some part of the whole network. Experimental data can also be used to infer yet unknown molecular interactions, propose existence of regulating proteins, etc. Some useful tools can handle such network data (Cline et al., 2007) and also some methods are developed that can help the search for missing interactions and to infer network topology (Aldridge et al., 2006; Andrecut et al., 2008; Fujita et al., 2007; Nelander et al., 2008). Since high throughput data is available for cell cycle of various organisms now, we can start to think about how to fuse these data to measurements on single gene perturbations to achieve a detailed understanding of the system. The computational identification of cell cycle related transcription factors (Cheng and Li, 2008; Wu and Li, 2008) is a promising initial result on these lines.

Spatial distribution of regulatory molecules

Another layer of complexity in cell cycle models is the matter of spatial distribution of regulatory molecules. Many crucial events happen in the nucleus and many molecules are moved in/out of the nucleus during the cycle. Still only a few cell cycle models consider compartmentalization of the cell (Chen et al., 2004; Yang et al., 2006). Even in compartmental models, diffusion and protein gradients are not considered, even though they might have important roles in regulation (Kholodenko, 2006). Simulation packages are available to deal with spatial distributions of proteins (Slepchenko et al., 2003), but experimental data on protein localization during the cell cycle is too spotty to give meaningful constraints for such models.

Time intensive simulations

A serious problem of spatial models with many interacting components could be the extensive computational time needed for simulations. Stochastic simulations face similar problems, in large models with many interacting components the calculations could slow down dramatically. In both cases, we need reliable methods for speeding up the simulations. In the case of stochastic simulations, there is a promising idea, based on the total quasi-steadystate assumption of enzyme kinetics (Borghans et al., 1996), for handling the coupled enzymatic reactions that are implied by the positive and negative feedback loops of the cell cycle network (Barik et al., 2008; Ciliberto et al., 2007). This and other methods (Gillespie, 2007) that decouple different time-scales can help us to handle stochastic noise in larger models in the future.

Qualitative models on dynamical properties of cell cycle

Other advances in the field of model analysis will extend the reach of bifurcation analysis, for tracking qualitative changes in the dynamics of a system on ODEs (Csikasz-Nagy et al., 2006), and sensitivity analysis, for identifying parameter combinations that crucially determine specific aspects of a simulation (Turanyi, 1990). Recently biological modeling has been enriched by some new concepts that help to decompose cell cycle models into sub-networks (Conradi et al., 2007), find the exact timing of cell cycle transitions (Lovrics et al., 2006) and check the irreversibility of these transitions (Ballarini et al., 2009). The last example uses a model-checking approach developed by computer scientists, and it is based on the automated verification of properties of the modeled systems that are encoded using some temporal logic formulae to verify if a system can reach a given state. This approach has opened some new and interesting research lines in biological modeling (Calzone et al., 2006; Heath et al., 2008; Mardare et al., 2004; Monteiro et al., 2008).

Combinatorial complexity

Some other interesting concepts have invaded biological modeling from computer science. Rule based modeling (Hlavacek et al., 2006; Regev and Shapiro, 2002) and especially various process algebras (Ciocchetta and Hillston, 2008; Priami and Quaglia, 2004b,a) were proposed to circumvent the problem of combinatorial complexity caused by modeling the nested network of multisite modification processes and multi-component complex formations, which are both relevant issues for cell cycle models (Kim et al., 2005; de Lichtenberg et al., 2005). The Beta Workbench modeling environment was developed to handle this type of problem with a biologically friendly computational language based on process algebra (Dematté et al., 2008; Dematte et al., 2008). This tool has been thoroughly tested and extended to handle large-scale models of cell cycle regulation.

4.6 Perspective

The core cell cycle module is regulated by several incoming signals and it drives several downstream events. The duty of this central controller is to process the information it receives and decide how to handle DNA replication and nuclear division. Current models use some parameters as incoming signals and can tell how this input determines the timing of cell cycle events. Some models already investigate how the circadian clock interacts with the cell cycle machinery (Zamborszky et al., 2007; Altinok et al., 2007; Bernard and Herzel, 2006) and how the cell cycle is regulated in response to checkpoint signals (Ciliberto et al., 2003; Alarcon et al., 2004; Iwamoto et al., 2008; Obeyesekere et al., 2004). These models are very detailed either on the

cell cycle machinery or on the signaling network, but comprehensive models that incorporate both control systems in detail do not exist yet.

Several models are available for pathways that signal to the cell cycle machinery the presence of nutrients, pheromones, stress inducing agents, etc. (Fuss et al., 2006; Klipp et al., 2005; Kofahl and Klipp, 2004; Schaber et al., 2006; Schoeberl et al., 2002; Wang et al., 2006). These could be merged with appropriate cell cycle models to reveal if our current knowledge of the signaling pathway, cell cycle network interactions is indeed complete.

Similarly, many other biological pathways have been proposed to interact with the cell cycle, such as polarized growth (Hayles and Nurse, 2001), the NF-κB pathway (Barre and Perkins, 2007), p53 regulation (Kastan and Bartek, 2004). While computational models also exist for these processes (Altschuler et al., 2008; Csikasz-Nagy et al., 2008a; Ihekwaba et al., 2007; Bentele et al., 2004), they have yet to be connected to the cell cycle models and to each other.

Another perspective is to step up from the single cell level and simulate how cell-to-cell interactions alter cell proliferation at the tissue level. This requires multi-scale parallel handling of the cell cycle controls within individual cells while simulating their interactions through signaling at the same time. For this problem we need, first of all, reliable cell cycle models for animal cells, desirably specific models of specific cell types, and in addition we need experimental measurements on the signaling between cells. Such detailed models are far in the future, but we already can learn from some models that take steps in this direction (Anderson et al., 2009; Chauhan et al., 2008; Ribba et al., 2006).

5 A mesoscale model of G1-S phase transition in liver regeneration

Synopsis

The liver regenerates and maintains its function and size after injury by counterbalancing cell death with compensatory cell division. During liver regeneration, injured sites release cytokines, which *primes* normally quiescent hepatocytes to enter the G1 phase of cell cycle. Growth factors stimulate the primed cells to overcome G1 checkpoint and enter cell cycle progression. Using a mesoscale approach, we have implemented the first mathematical model that describes cytokine-induced dedifferentiation of hepatocytes and the subsequent initiation of DNA synthesis (G0-G1-S phase of the cell cycle). The model accurately reproduces experimentally measured kinetics of various signaling intermediates and DNA synthesis in hepatocytes for varying degrees of liver damage, in both wild type and knockout backgrounds. Liver regeneration is known to be a robust process, as liver mass reconstitution still occurs in various knockout mice (albeit with different kinetics). We analyze the robustness of the model using methods of control analysis. Moreover, we discuss the system's bandpass filtering properties and delays, which arise from feedbacks and nested feed-forward loops.

5.1 Introduction

Mathematical modeling of cellular processes has been pioneered by Reinhardt Heinrich (Heinrich and Schuster, 1996). In order to quantify the transient dynamics during liver regeneration, we apply tools of control analysis developed by Heinrich et al. (2002) for signaling cascades.

Being the main detoxifying organ of the body, the liver is susceptible to damage by ingested toxins. In order to maintain its architecture and function, nature provides the liver with a remarkable capacity to regenerate after injury by counterbalancing cell death with compensatory cell division. Such cell division is mainly carried out by the major hepatic cell type, i.e., hepatocytes, and is complete within one week in mice. Experimentally, liver regeneration is often initiated by surgical removal of a part of the liver, this procedure is also referred to as partial hepatectomy (PH). Surgical resection[] of two thirds of the liver (2/3 PH) serves as a popular

model for synchronous cell division *in vivo*, and has been extensively studied experimentally.

In a normal adult liver, cells rarely divide and are considered to be in a quiescent state, i.e., in the G0 phase of the cell cycle. However, after PH, hepatocytes rapidly enter the cell division cycle, which consists of four phases: G1-S-G2-M. During S phase, DNA replicates, and the M phase is characterized by chromosomal separation and cell division. S and M phases are separated by two gap phases, G1 and G2, during which cells prepare for the next phase. In the context of liver regeneration, the transition from G0 to G1 is often called *priming*, while the subsequent passage into S phase (G1-S transition) is usually termed *progression* (Fausto, 2000).

The entry of quiescent hepatocytes into the cell cycle is regulated by multiple pathways, including those induced by cytokines (e.g., IL-6 and TNF-α) and growth factors (e.g., HGF and HB-EGF). Priming is mainly governed by tumor necrosis factor (TNF-α) and interleukin-6 (IL-6), which induce a variety of immediate early genes (IEG) including c-jun, c-fos, and c-myc. These early priming events are necessary but not sufficient for the initiation of DNA synthesis. Progression into S phase further requires cellular stimulation by growth factors, which are transcriptionally induced in the late priming phase (Fausto, 2000; Taub, 2004).

Cytokines and growth factors coordinately induce the expression of cyclins, which are the master regulators of the G1-S transition. Cyclin D, whose expression is directly regulated by growth-factor and cytokine-induced intracellular signaling pathways, appears in the mid-G1 phase (Taub, 2004). Cyclin D/cdk4 complexes phosphorylate a variety of cellular substrates leading to the expression of target genes necessary for progression into S phase. One of these target genes, cyclin E, which appears in the late G1 phase and early S phase, is the main initiator of DNA replication. The activities of cyclin D and cyclin E are inhibited by a family of cyclin associated inhibitors (e.g., p21 and p27) and fine-tuned by regulated proteolysis (Malumbres and Barbacid, 2005).

There are several models of the G1-S transition in cultured cell lines (Kohn, 1998; Aguda and Tang, 1999; Qu et al., 2003b; Novak and Tyson, 2004; Swat et al., 2004; Haberichter et al., 2007). Here we present a model of the initiation of synchronous cell division during liver regeneration *in vivo*. Liver regeneration is orchestrated by a complex interplay between various intra- and extracellular signaling pathways, which makes it difficult to include all molecular species. Thus, we implement a mesoscale model to explain the G0-G1-S phase transition after PH in the mouse. Mesoscale model refers to a higher level of description where we lump together some species and reactions. The model is constructed to reproduce experimentally measured kinetics of various signaling intermediates and DNA synthesis in hepatocytes for varying degrees of liver damage in both wild type and knockout backgrounds. We further analyze the robustness and the filtering properties of the model and find that the model exhibits a bandpass filter arising from feedbacks and nested feed-forward loops.

5.2 The model

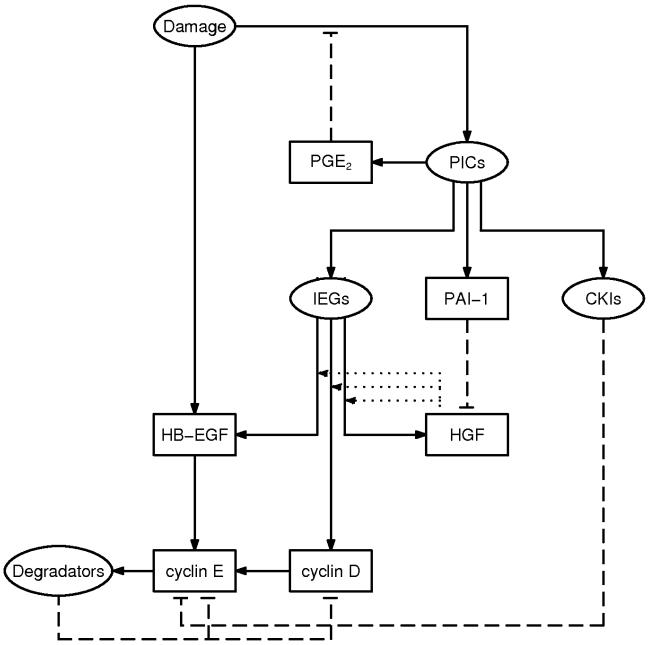

Figure 5.1:
Graphical scheme for G0-G1-S transition during liver regeneration. G0-G1-S transition is driven by two pathways: (1) Cytokine induced priming. *Damage* induces *PIC* (IL-6, TNF-α). *PIC* further induces synthesis of *IEG* (c-jun, C/EBP-β) and *PAI*-1, and activates inhibitors like *PGE*2 and *CKI* (p21, p27), simultaneously. *IEG* induces transcription of growth factors *HGF* and *HB-EGF*, and also its inhibitor *PAI*-1. (2) Growth factor induced pathway: *Damage*, *IEG* and *HGF* produce the growth factor *HB-EGF*. *HB-EGF* further induces *cyclin E* which is also stimulated via *cyclin D*1, marking G1-S transition. Ultimately *CKI* and other degradating signals like *deg* lead to degradation of *cyclin E*. Species inside rectangles represent single entities while species inside ellipses comprise a group of species with similar kinetics.

Based on the existing literature and the time series data available on PH experiments in mice, we construct a mesoscale model focussing on the initiation of DNA synthesis (Fig. 5.1). Arrows in the figure typically represent complete signal transduction pathways from extracellular stimulation to gene induction. We justify this drastic simplification with the assumption that transcription occurs at a much slower rate than post-transcriptional signal transduction, so that the dynamical behavior of the system can solely be described in terms of changes at transcriptional level. We choose Hill equations to represent known features of signal transduction cascades such as saturation and ultrasensitivity. We neglect ultrasensitivity (by setting the Hill coefficient to 1) and/or saturation (by assuming a high half saturation point) if these features are not necessary to describe experimentally measured time courses during liver regeneration. We assume that protein degradation always follows linear kinetics.

The model input is liver damage induced by 2/3 PH. Regarding the output, most experimentalists

analyzed the time course of DNA synthesis after PH as a readout for liver regeneration. The time course of DNA synthesis agrees well with that of cyclin E activity during liver regeneration (Wüstefeld et al., 2000). Moreover, cyclin E activity is highly sensitive to the degree of liver damage (as is DNA synthesis), while no such sensitivity is observed for cyclin D (Mitchell et al., 2005a). Thus, we use cyclin E activity (i.e. the amount of free cyclin E) as the output variable in our model. The model consists of 12 rate equations and 40 parameters (Refer to appendix in Chauhan et al. (2008). Details regarding the choice of kinetic parameters using experimental data are provided as supplementary material in Chauhan et al. (2008). In the following we summarize the basic state variables described by our model.

Damage The default input stimulus in our model is the liver damage caused by 2/3 PH in a mouse. Two types of PH-induced damage are thought to be mainly involved in the induction of downstream signaling and hepatocyte cell division: (i) Cell necrosis at injured sites triggers inflammatory responses, e.g., the release of pro-inflammatory cytokines (Leu et al., 2003). (ii) Liver mass depletion also seems to be involved in the induction of hepatocyte cell division (Lambotte et al., 1997). Markers of liver cell necrosis such as serum aspartate aminotransferase and alanine aminotransferase remain significantly elevated until 40 hours after 2/3 PH (Leu et al., 2003). Liver mass increases steadily after 2/3 PH, with mass restoration being essentially complete after 96 hours (Blindenbacher et al., 2003; Taub, 2004). Based on these observations, we simulate a 2/3 PH experiment by assuming that the input in our model, termed *Damage*, decays exponentially with a half-life of 20 hours.

PIC (Pro-Inflammatory Cytokines) and PGE2 (Prostaglandin E_2) Experimental studies reveal that tissue damage due to PH triggers the release of lipopolysaccharides (LPS) into the blood stream. LPS is known to induce the release of TNF-α from Kupffer cells. TNF-α in turn activates the intracellular transcription factor NF-κB in Kupffer cells, and thereby induces transcription of IL-6 (Taub, 2004). In our model, we have lumped up IL-6 and TNF-α in one variable, the pro-inflammatory cytokines (PIC) (Fig. 5.1). PICs are assumed to be directly induced by *Damage*.

IL-6 release during liver regeneration after PH was shown to be transient (Liao et al., 2004). This seems to be due to the fact that IL-6 induces PGE2 expression in Kupffer cells which regulates the production of TNF-α and IL-6 in a negative feedback loop (Goss et al., 1993). Thus we assume in our model that PIC is inhibited via PGE_2.

IEG (Immediate Early Genes) The earliest genes expressed in hepatocytes after PH, referred to as immediate early genes, are regulated by Kupffer-cell-derived PICs and appear within 1-2 hours. Among these immediate early genes there are several transcription factors including c-jun, c-fos, STAT3 and C/EBP-β (Taub, 2004). We lump these early transcription factors into a combined species referred to as IEG owing to its similar, transient kinetics of expression and

phenotypically similar knockouts (Cressman et al., 1996; Greenbaum et al., 1998; Behrens et al., 2002).

PAI-1 (Plasminogen Activator Inhibitor) PAI-1 functions as a negative regulator of liver regeneration by inhibiting the proteolytic maturation of HGF (Michalopoulos and DeFrances, 1997; Taub, 2004). PAI-1 is induced early after PH and was shown to be regulated by TNF-α and IL-6 (Healy and Gelehrter, 1994; Shimizu et al., 2001). Therefore we model *PAI* to be induced by *PIC*.

CKI (Cyclin dependent kinase inhibitors) In the model, *CKI* represents the cip/kip family of cyclin dependent kinase inhibitors (CKI) which include p21 and p27. They bind to a wide range of cyclin/CDK complexes and stoichiometrically inhibit cyclin activity (Malumbres and Barbacid, 2005). Since the expression of p21 is mediated by IL-6 via STAT3 (Wüstefeld et al., 2000), we model *CKI* to be directly induced by *PIC*.

HGF (Hepatocyte Growth Factor) Early priming events are necessary but not sufficient for the initiation of DNA synthesis (Fausto, 2000). Progression into S phase further requires cellular stimulation with the growth factors such as HGF and HB-EGF. HGF, a potent stimulator of DNA synthesis in hepatocytes (Michalopoulos and DeFrances, 1997), is induced by several factors including pro-inflammatory cytokines (IL-6, TNF-α) via immediate early transcription factors such as C/EBP-β (Liu et al., 1994), all of which are elevated during immediate early phase. HGF in turn activates a variety of signaling molecules (Ras, Erk, Akt) and IEGs (c-jun, c-fos) (Borowiak et al., 2004). Many of these signal transduction molecules and transcription factors seem to be synergistically activated by cytokines and growth factors (Taub, 2004). Based on these data, we assume that HGF is induced by *IEG*. *HGF* is modeled to further amplify *IEG* induced gene expression, and thereby positively feeds back on its own expression. Additionally, we assume in the model that *HGF* synthesis is blocked by *PAI*-1, as the proteolytic maturation of HGF was experimentally shown to be inhibited by PAI-1 (Shimizu et al., 2001).

HB-EGF (Heparin Binding Endothelial Growth Factor) HB-EGF is an important EGF family growth factor whose expression correlates well with cyclin E activity and DNA synthesis during hepatic regeneration (Mitchell et al., 2005a). HB-EGF expression is induced by the Raf/MAPK pathway (McCarthy et al., 1995), and is therefore expected to be activated by HGF. However, in contrast to HGF and priming genes, HB-EGF expression is known to be sensitive to the amount of liver excised during PH (Mitchell et al., 2005a). These data suggest that HB-EGF expression is regulated by early upstream damage sensing pathways as well as by HGF. In order to model HB-EGF's ability to act as a sensor for the degree of damage, we assume in the model that HB-EGF synthesis requires simultaneous inputs from both *Damage* and *HGF*.

Cyclin D and Cyclin E Cyclins and their regulatory kinases (cdks) regulate the progression of the cell cycle from G1 phase (D cyclins, cdk4,6) into S phase (cyclin E/cdk2). Cyclin D and cyclin E complexes are important for G1-S transition because they phosphorylate Rb and E2F factors, which in turn, lead to expression of genes necessary for cell cycle progression. Cyclin D is induced by growth factors via Ras/Raf/Erk signaling (Aguda, 2001). Thus, we model *cyclin D* to be coordinately regulated by HGF and IEG. Cyclin E expression is known to be positively regulated by cyclin D, and can be induced directly by growth factors as well (Aguda, 2001). In particular, it was shown that cyclin E activity correlates well with that of HB-EGF expression during liver regeneration (Mitchell et al., 2005a). Owing to these data, we assume in the model that cyclin E synthesis is induced by *cyclin D* and $HB\text{-}EGF$ in an additive manner. As discussed before, our mesoscale approach implies that we lump together many individual steps. Owing to the complexity of Rb/E2F regulation during G1-S transition (Swat et al., 2004), we do not model the dynamics of Rb and E2F explicitly, but incorporate them implicitly within the cyclin D and cyclin E activation pathways.

CKIs such as p21 are known to be stoichiometric inhibitors of cyclin E (Malumbres and Barbacid, 2005). Hence, we have modeled CKI-inhibition of cyclin E by a reversible association of both proteins into an inactive complex. Cyclin D/CDK complexes are also known to bind to CKIs, and thus complex formation between CKIs and cyclin D are considered in the model as well.

Degradator (deg) The expression levels of cyclin D and E are tightly regulated by ubiquitin-dependent proteolysis. Cyclin E degradation was reported to depend at least in part on cyclin E activity, and cyclin E degradation seems to occur in a negative feedback loop fashion (Welcker et al., 2003). Cyclin D was also shown to be efficiently degraded during S phase (Guo et al., 2005), but the underlying regulatory mechanisms remain obscure. For simplicity, we assume in our model that *cyclin D* degradation is also governed by the S phase master regulator, namely, $cyclin\ E$. Thus $cyclin\ E$ induces the expression of deg in the model, and these in turn enhance the degradation of both $cyclin\ E$ and $cyclin\ D$ in a negative feedback loop.

5.3 Results

Having established the topology of the network for G0-G1-S phase transitions of cell cycle during liver regeneration, we adjust the kinetic parameters (see Appendix and supplementary data from Chauhan et al. (2008) for details of simulations) such that the model represents the dynamic patterns of the hepatic responses to varying external stimuli (2/3 vs 1/3 PH) and perturbations such as knockouts.

5.3.1 Kinetics and extent of DNA synthesis

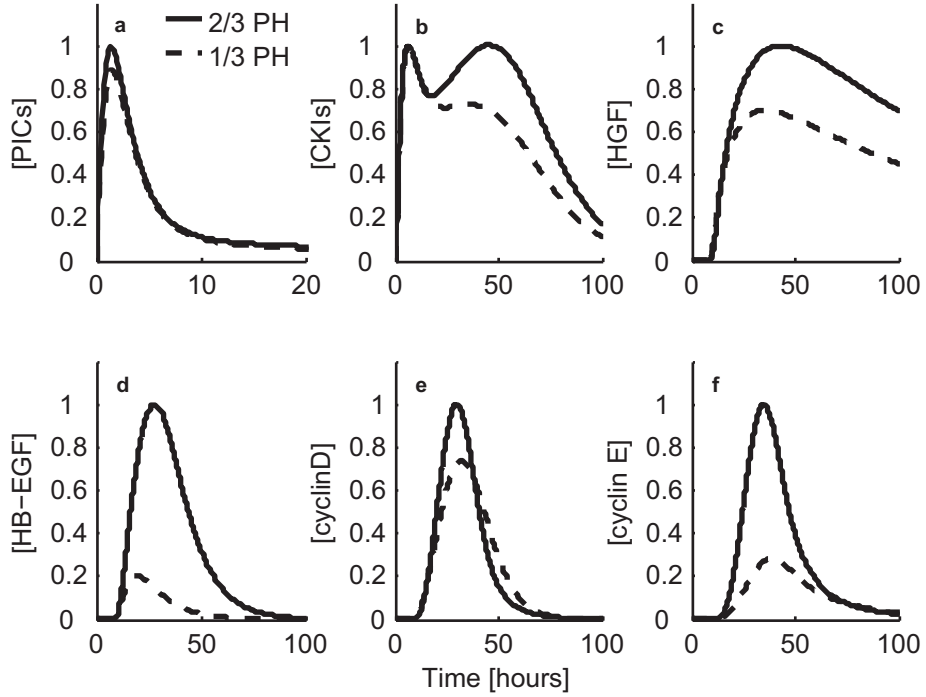

Figure 5.2: Simulations of dynamic variables in 2/3 PH (solid curve) and 1/3 PH mouse (dashed curve).

Numerical simulations of the model are carried out to resemble the damage caused by 2/3 PH. Most experimental studies showed that priming factors like IL-6, IEG, PAI-1 rise early after PH and are back to basal levels within 10-15 hours (Greenbaum et al., 1998; Li et al., 2002; Behrens et al., 2002; Fausto, 2000). The corresponding model species (*PIC*, *IEG* and *PAI*) reflect such transient behavior reasonably well (*PIC* is shown as an example in Fig. 5.2a).

p21 mRNA has been experimentally observed to start rising slowly at 1-2 hours and to remain elevated for 48 hours (Behrens et al., 2002). *CKI*, representing p21 in the model, reproduce these temporal dynamics (Fig. 5.2b).

Among growth factors, active HGF levels have been shown in experiments to be below detectable levels for several hours after PH. Then there is a strong increase in the levels of HGF and subsequently a gradual decrease takes over (Pediaditakis et al., 2001; Shimizu et al., 2001). HB-EGF on the other hand, is known to be expressed before HGF with a peak at 24 hours followed by a fast decline (Fausto et al., 2006). *HGF* and *HB-EGF* in the model reproduce these temporal dynamics correctly (Fig. 5.2c, 5.2d).

The major regulators cyclin D and cyclin E are observed experimentally to have a 12-16 hours delayed peak appearing between 36-48 hours followed by a decline to near zero levels (Liao

et al., 2004). Our numerical simulations mimic these observations (Fig. 5.2e, 5.2f).

Various 1/3 vs. 2/3 PH experiments have shown that the magnitude of DNA synthesis is sensitive to the amount of liver excised while the timing of DNA synthesis remains essentially unchanged (Lambotte et al., 1997; Mitchell et al., 2005a). We carry out numerical simulations of 1/3 PH and 2/3 PH by varying the magnitude of *Damage*, I_0, in the model, and use cyclin E activity (i.e., the amount of free cyclin E) as a measure of DNA synthesis (see model derivation). In accordance with the experimental studies, the simulated amplitude of *cyclin E* activity is quite different for 1/3 PH and 2/3 PH while the timing stays fairly constant (Fig. 5.2f).

Mitchell et al. (2005a) compared effects of 1/3 PH vs. 2/3 PH on protein expression during priming and progression phases of liver regeneration. HB-EGF expression was found to be sensitive towards the amount of damage exerted on the liver, with greatly reduced levels at 1/3 PH unlike *PIC*, *HGF* and *cyclin D*. The model reproduces this decrease in *HB-EGF*, while it shows insensitive behavior towards the amount of damage in the case of *PIC*, *HGF* and *cyclin D* (Fig. 5.2a, 5.2c, 5.2e), which is in accordance with experimental data.

Thus the model reflects the experimentally established fact that expression of priming factors such as PICs and IEGs alone is not sufficient for cyclin E activation and DNA synthesis. The sensitivity of both *HB-EGF* expression and *cyclin E* activity to the degree of damage supports that HB-EGF is crucial for progression through G1-S transition and links priming with progression (Mitchell et al., 2005a).

5.3.2 Simulations of knockouts

Knockouts of important positive and negative regulators were modeled by setting their synthesis to zero, or, in the case of lumped factors, by decreasing their synthesis (Fig. 5.3) (see Appendix and supplementary data). The resulting time courses of cyclin E activity are compared to experimental measurements of DNA synthesis.

Cressman et al. (1996) measured the kinetics of DNA synthesis for IL-6 knockout mice. Owing to the redundancy of species (IL-6 and TNF-α) in *PIC*, we modeled $PIC^{(-/-)}$ by decreasing the synthesis rate of *PIC*. In accordance with the experimental studies (Cressman et al., 1996), the simulated $IL\text{-}6^{(-/-)}$ system exhibits reduced peak cyclin E activity when compared to wild type, but no change in the timing of peak cyclin E activity. Wüstefeld et al. (2000) engineered transgenic mice which overexpress soluble IL-6 receptor/gp80 to analyze the effect of hyperstimulation with IL-6. They observed a delayed onset and peak of DNA synthesis and a reduced peak. To simulate overexpression, we increase the synthesis rate of *PIC*. $PIC^{(oe)}$ shows a markedly decreased DNA synthesis with the peak shifted by 19 hours (Fig. 5.3a).

Recall that *IEG* is a lumped variable describing various transcription factors. STAT3 knockout

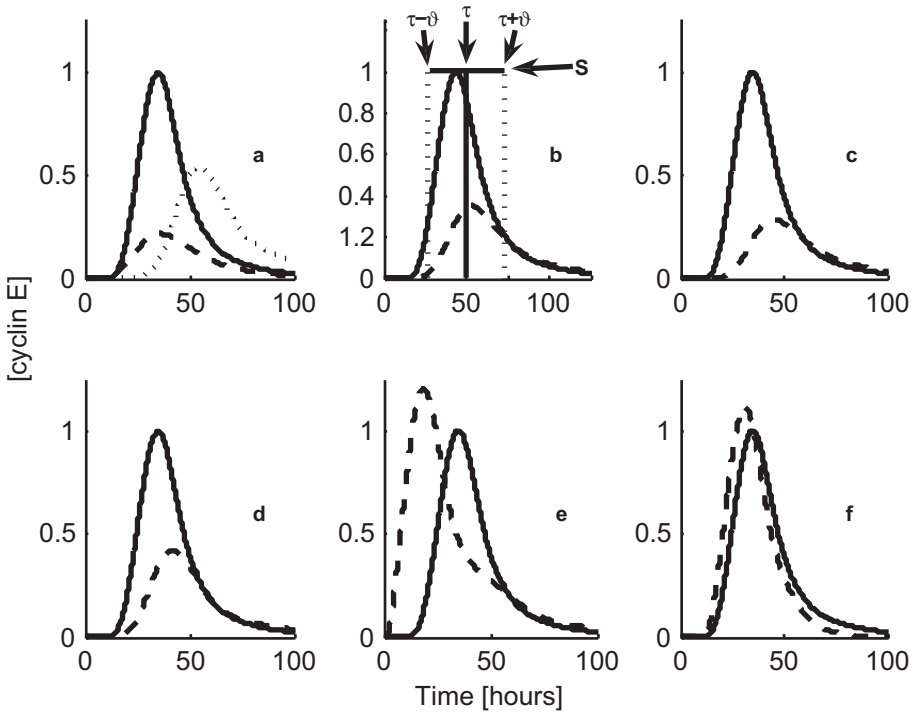

Figure 5.3:
Simulations of knockouts. (a) solid line: wild type *IL-6*, dashed line: *IL-6(-/-)*, dotted line: *IL-6* hyperstimulation; (b) solid line: wild type *IEG*, dashed line: *IEG(+/-)*, τ, S and ϑ: signal time, signal amplitude and signal duration as defined by Heinrich et al. (2002); (c) solid line: wild type *HGF*, dashed line: α HGF antibody; (d) solid line: wild type *HB-EGF*, dashed line: *HB-EGF(-/-)*; (e) solid line: wild type *PAI-1*, dashed line: *PAI-1(-/-)*; (f) solid line: wild type *CKI*, dashed line: *CKI(-/-)*

.

(Li et al., 2002), c-jun deletion (Behrens et al., 2002) and targeted disruption of the C/EBP-β gene (Greenbaum et al., 1998) can be simulated by decreasing the synthesis rate of *IEG*. In all these experimental knockouts, the amplitude of DNA synthesis was markedly reduced while peak timing stayed fairly unchanged. The amplitude of *cyclin E* synthesis in $IEG^{(+/-)}$ is greatly reduced to about one-third with no marked change in peak timing, as observed in the experiments (Fig. 5.3b).

HGF signalling was blocked experimentally by knocking out the HGF receptor met (Borowiak et al., 2004) or by an α-HGF antibody (Burr et al., 1998). In both cases, a drastic reduction in *cyclin E* synthesis was observed. We model HGF depletion (α-$HGF^{(+/-)}$) by decreasing the synthesis rate of *HGF* and find a dramatically reduced *cyclin E* synthesis as shown in the experiments (Fig. 5.3c).

Mitchell et al. (2005a) performed 2/3 PH in *HB-EGF* knockout mice and observed a delayed and significantly reduced DNA synthesis. Due to redundant EGF family members such as TGF-α, *HB-EGF* synthesis could not be reduced to zero. *HB-EGF* knockout is mimicked by lowering

the synthesis rate of *HB-EGF*. The simulated *cyclin E* activity in the model is significantly reduced which correlates well with the experiments (Fig. 5.3d).

Experimental studies reveal that negative regulators delay DNA synthesis during liver regeneration, but have relatively minor effects on the amplitude (Shimizu et al., 2001; Jaime et al., 2002; Luedde et al., 2003; Hayashi et al., 2003). Simulations of *PAI*-$1^{(-/-)}$ and $CKI^{(-/-)}$ show a forward shift in timing of *cyclin E* synthesis with no marked difference in the amplitudes of wildtype and knockout mice, as observed experimentally (Fig. 5.3e, 5.3f).

Taken together, we can conclude that knockouts of the negative regulators of G1-S transition during liver regeneration seem to modulate the timing of DNA synthesis much more than the amplitude of synthesis. On the contrary, positive regulators seem to affect the amplitude, but have much less effect on the timing of synthesis. This indicates that positive signaling mainly determines the extent of DNA synthesis, whereas negative signaling (via p21 and PAI-1) sets the timing of DNA synthesis. In the following section[], we systematically study how amplitude and timing are controlled.

5.3.3 Sensitivity analysis of the model and robustness of liver regeneration

Liver regeneration is known to be a very robust process, as liver mass is still capable of reconstituting even in the absence of important species. Therefore we expect that the total *cyclin E* activity, as a measure of total DNA synthesis, to be a robust property of the model. We analyze the control of integrated response, signal amplitude and signal time with respect to changes in biochemical parameters. More specifically, we perturb the synthesis rates of each individual species over a 100-fold range and analyzed the sensitivity of *cyclin E* in terms of its signal amplitude (S) and signal time (τ), as defined by Heinrich et al. (2002) (see Fig. 5.3b).

With 100-fold perturbations, changes in cyclin E amplitude and integrated response were below 10-fold in all but one cases. Since the rate of synthesis of *deg* is immediately downstream to *cyclin E*, the time course of *cyclin E* activity is most sensitive to its perturbations (Fig. 5.4a). Perturbations in the synthesis rates of *PGE*2 (Fig. 5.4b), *PAI* (Fig. 5.4c), and *IEG* (Fig. 5.4d) led to less than 10-fold changes in cyclin E integrated response and amplitude. *HB-EGF*-dependent rate of synthesis of *cyclin E* (Fig. 5.4e), *Damage* dependent rate of synthesis of *HB-EGF* (Fig. 5.4f), rate of synthesis of *HGF* and rate of synthesis of *CKI* (Fig. 5.4g) show less than 7-fold changes over a 100-fold range of perturbations. In the other cases the amplitude and integrated response change less than 2-fold despite 100-fold parameter changes as summarized in Fig. 5.4h.

In terms of signal time (τ) the system seems to be even more insensitive to parameter changes. With 100-fold change of synthesis rates, signal times is changed by less that 2-fold in all cases.

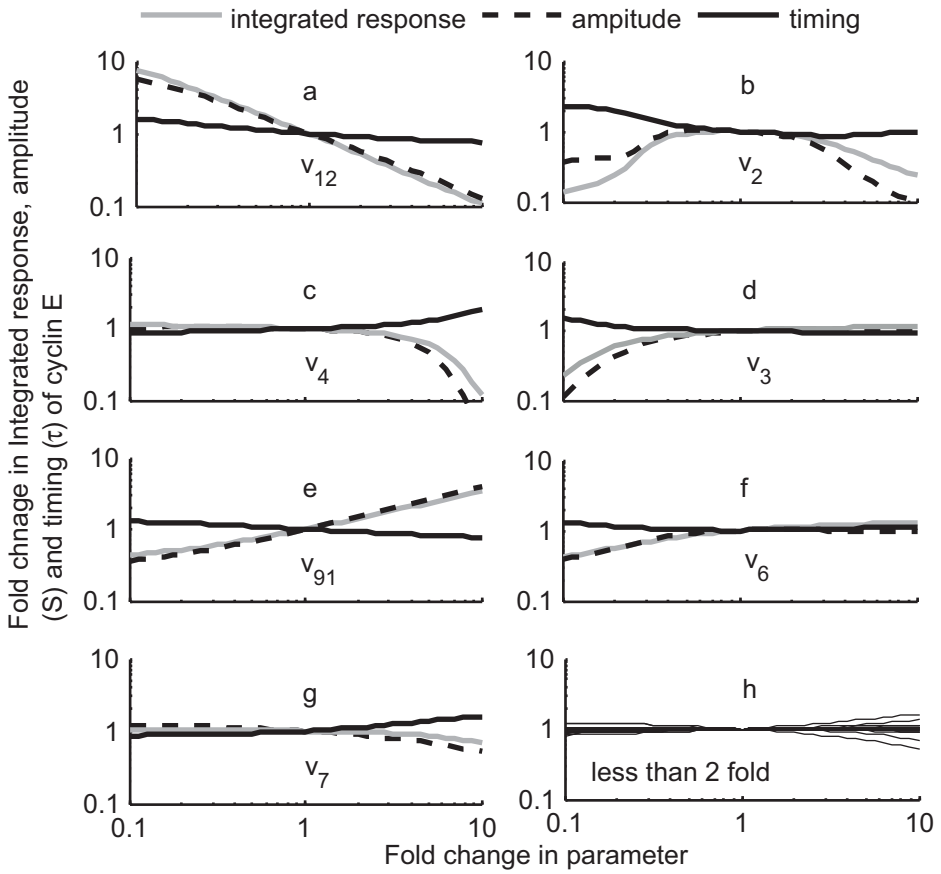

Figure 5.4:
Fold change in integrated response, signal amplitude (S) and signal time (τ) of *cyclin E* with fold change in parameters. (a) Rate of *cyclin E* mediated *Degradator* synthesis; (b) rate of *PGE*2 synthesis; (c) rate of *PAI*-1 synthesis; (d) rate of *IEG* synthesis; (e) rate of *HB-EGF*-mediated *cyclin E* synthesis; (f) rate of *HB-EGF* synthesis; (g) rate of *CKI* synthesis; (h) remaining 9 synthesis rates.

In summary, the model simulations confirm the robustness of liver regeneration. Only in a few cases, 100-fold changes of 16 synthesis parameters lead to drastic changes of the signal amplitude and the integrated response.

5.3.4 Filtering properties of the model

Sensitivity analysis of the model gave insights into the modulation of timing and amplitude at each stage of the model leading to a balanced and robust DNA synthesis. We further study the filtering properties of the model output of *cyclin E* by changing the *Damage* input. We vary the magnitude of *Damage* (I_0) in such a way that the total amount of *Damage* (area under curve for *Damage*) remains constant while adjusting the decay time. High magnitude I_0 means

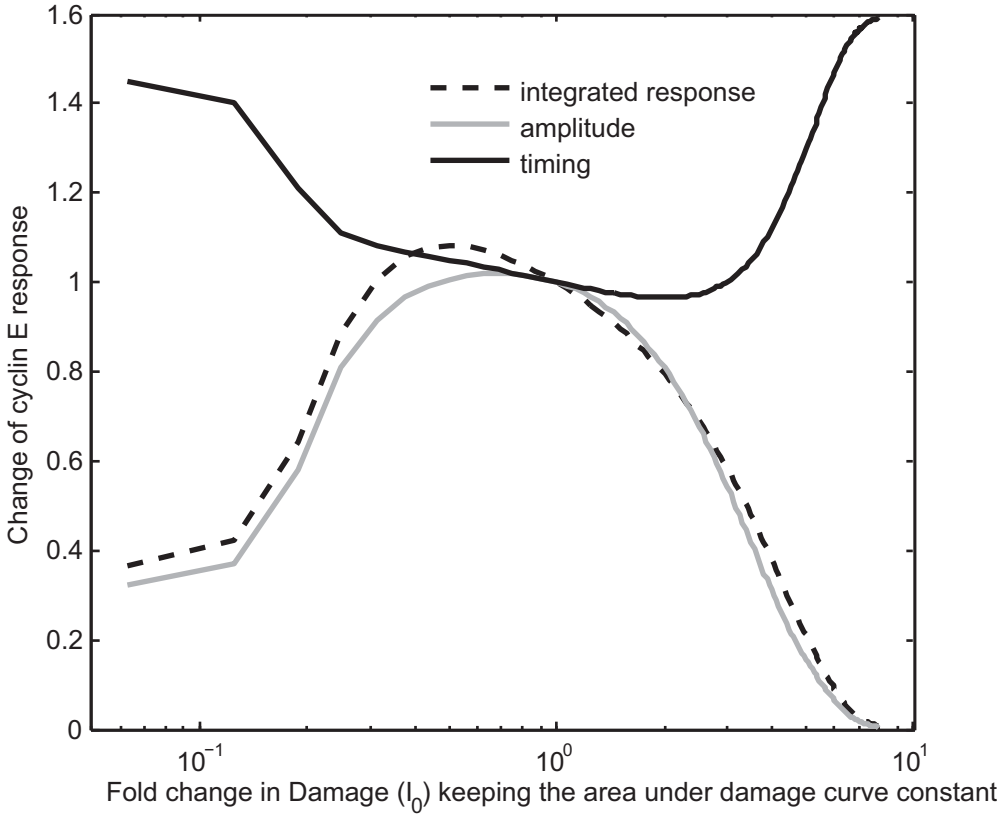

Figure 5.5: Input/output relationship of the model with changes in magnitude of *Damage* (I_0)

a large but short lived *Damage*, and a low magnitude I_0 refers to a weak and prolonged *Damage*. Interestingly, too sharp and too prolonged Damages were filtered out, and thus the output (area under curve for *cyclin E*) showed the attributes of a *bandpass filter* (Fig. 5.5).

The model architecture contains various feed-forward loops. This means that the common input regulator (*Damage*) regulates the output (*cyclin E*) via independent (multistep) branches. This is a generalization of classical feed-forward loops discussed by Dekel et al. (2005). We found that the nested feed-forward loops in the *PIC* mediated pathway including the incoherent feed-forward loop from *PIC* to *HGF* via *PAI* and the incoherent feed-forward loop from *PIC* to *cyclin E* via *CKI* is responsible for filtering out sharp short-lived inputs. On the other hand, weak and long-lived inputs are filtered out due to ultrasensitivity involved in *HB-EGF* production and *HB-EGF* mediated *cyclin E* induction (see equations and supplementary data).

Compared to the amplitude, the timing of *cyclin E* synthesis is much less sensitive to changing of *Damage* (Fig. 5.5). This confirms the experimental observation that the timing of hepatocyte DNA synthesis after PH is robust. When PH treated rat hepatocytes are transplanted into PH treated mouse liver, rat hepatocytes in mouse liver display rat kinetics despite the new surround-

ing. Thus the model can also simulate cell autonomous behavior of timing of hepatocyte entry into DNA synthesis (Weglarz and Sandgren, 2000).

5.4 Discussion

In this chapter, we presented a mesoscale model of liver regeneration where we lumped similarly acting species into single variables, but did not consider individual mRNA and protein concentrations. This approach allows us to accurately describe the complex dynamics of liver regeneration (see Figs. 2 and 3) even though only few quantitative experimental informations were available. The model can be used to make experimentally testable predictions. In particular, the model can be used to predict how the dynamics of DNA synthesis during liver regeneration is altered in transgenic mice which overexpress key mediators of liver regeneration. In particular, it turns out that positive signaling governs the extent of DNA synthesis and inhibitors control the timing.

Liver regeneration is a remarkably robust process, as mass reconstitution accurately occurs in most cases, even if the key players of liver regeneration are knocked out. Liver mass reconstitution mainly occurs by compensatory cell division. Therefore we expected integrated response of Cyclin E activity to be a robust property of the model, as it reflects the total amount of DNA synthesized during liver regeneration. Our simulations shown in Fig. 4 revealed that the integrated response of Cyclin E is indeed relatively insensitive to parameter changes and explaining the observed robustness of liver mass reconstitution. We also found that the amplitude and the timing of DNA synthesis are relatively unaffected by parameter changes. This is also in accordance with experimental studies, as hepatocytes initiate DNA synthesis during liver regeneration in a highly synchronous manner, even though individual cells are likely to significantly differ from each other.

Our analysis of the model's filtering properties revealed that the model acts as a bandpass filter, that is, it suppresses short, high amplitude signals and long, low amplitude signals (Fig. 5). Such bandpass filtering appears to be physiologically relevant in the context of liver regeneration, because very strong liver damage suppresses the DNA synthetic response rather than increasing it (Bucher and Swaffield, 1964). More detailed analyzes suggested that incoherent feed-forward loops, i.e., simultaneous activation and inhibition in parallel, are responsible for filtering out short, high amplitude pulses. More specifically, it seems that inhibitory molecules such as p21 and PAI-1 are already expressed in response to short inputs, while activators of cell division require longer stimulation. These findings might also be of broader interest, as incoherent loops are involved in a variety of physiological processes. For example, cell death signals such as TNF-alpha trigger prosurvival signals such as NF-κB in addition to prodeath signals (Stehlik et al., 1998). The short-pulse filtering properties of incoherent feed-forward loops discussed in this paper might help cells to ignore erroneous short inputs, but to execute cell fate decisions in response to sustained stimulations.

In this work, we used a top-down approach to model liver regeneration. This framework will allow us to model currently lumped species in more detail in the future. For example, the immediate early genes (IEG) could be modeled as a network of transcription factors, which will allow direct comparison of the model with microarray data of liver regeneration. Additional extensions such as a complete description of the whole cell division cycle, as well as of spatial and stochastic effects, will enable us to determine why some hepatocytes enter a second round of cell division, while others do not.

6 The integrated mammalian cell cycle model of regenerating liver

Synopsis

The whole process of cell division in mammalian cells is mainly orchestrated by cell cycle dependent oscillations in the levels of cyclins. Different cyclins are produced at different cell cycle stages with additional controls imposed on them by various regulating mechanisms which govern distinct cell cycle events. G1-S cyclins which trigger DNA synthesis are discussed in chapter 2 and 2. The rise of G1-S cyclins is accompanied by the appearance of Cyclin A. Later mitotic events are controlled by Cyclin B . Mitotic transition is promoted by abrupt accumulation of active Cyclin B controlled by multiple redundant feedback loops at the transcriptional posttranslational and degradation levels. However, the relative contribution of these separate feedback loops to the decision to enter mitosis is currently under the scanner. Mitotic transcription factors like FoxM1 are being implicated more and more in the maintenance of G2 phase. Also, Cdh1 dependent proteolytic degradation of mitotic cyclins, which is more known for controlling the G1 phase, is being implicated in the control of G2 phase and G2-M transition. Exit from mitosis is controlled by Cyclin B degradation and mitotic exit is rendered irreversible due to systems-level feedbacks imposed by Wee1 and Cdc25 at early G1 phase. This chapter presents an integrative model of mammalian cell cycle focusing on Cdh1 at the core of cell cycle machinery and importance of FoxM1 and Wee1 in the mitotic machinery.

6.1 Introduction

In the presence of pro-inflammatory cytokines (PICs), hepatocytes quit a quiescent state, denoted G0, and enter *priming*. Primed cells in the early G1 phase are then driven by growth factor (GFs) to pass the restriction point, which is a point of no return beyond which they are irreversibly engaged in the cell cycle and do not require the presence of growth factors to complete mitosis (Taub, 2004). Progression in the cell cycle is controlled by the sequential activation of a family of cdks, which allow an ordered succession of the cell cycle phases G1, S, G2, and M (Morgan, 2007). The cdk proteins are active only when forming a complex with their corresponding cyclin. The Cyclin D-Cdk4/6, Cyclin E-Cdk2, Cyclin A-Cdk2, and Cyclin B-Cdk1 complexes promote,

respectively, progression in G1, the transition to S from G1, progression in S and G2, and finally the G2-M transition, allowing entry into mitosis (Morgan, 2007). Cdk regulation is achieved through a variety of mechanisms that include association with cyclins and protein inhibitors, phosphorylation/dephosphorylation, and cyclin synthesis or degradation (Morgan, 2007).

A number of theoretical models for the cell cycle have been proposed which are discussed in more detail in chapter ẽfChapter4. Initially, these models pertained to the early cell cycles in amphibian embryos (Tyson, 1991; Goldbeter, 1991; Novak and Tyson, 1993; Sha et al., 2003; Pomerening et al., 2003) which consist of only two phases, interphase and mitosis (Murray and Kirschner, 1989). Chen et al. (2004) later proposed a detailed computational model for the yeast cell cycle, which accounts for the behavior of a large number of mutants. Theoretical models were subsequently proposed for portions of the mammalian cell cycle, particularly the G1-S transition and the restriction point (Aguda and Tang, 1999; Qu et al., 2003a,b; Swat et al., 2004; Novak and Tyson, 2004). A generic model for the eukaryotic cell cycle has also been presented Csikasz-Nagy et al. (2006).

We still lack a detailed, integrative model coupling the different cyclin-cdk complexes that control the successive phases of the mammalian cell cycle, which would be capable of describing their sequential activation. Models of this sort were proposed for the yeast cell cycle in which a key role is played by cell growth; in those models mitosis is controlled by cell mass (Chen et al., 2004). In mammalian cells, cell mass is not a determining factor for the control of cell cycle. Also, modeling cell cycle regulation in mammalian cells is most daunting task, since it involves multiple control mechanisms that hold cells back from proliferation. Different types of mammalian cells are physiologically different from one another which makes the task of modeling mammalian cells even more difficult. Adding to this complexity, *in vitro* systems of artificially dividing cells are obtained under non-comparable experimental conditions, and different artificial cell cycle synchronization procedures. Liver regeneration being a highly synchronized proliferating system, provides the possibility to analyze the cell cycle of naturally synchronized cells in the whole animal.

The availability of comparatively well studied G1-S phase experimental models of liver regeneration in rodents allowed us to model a cytokine and growth factor mediated G1-S transition in regenerating hepatocytes in the previous chapter (Chauhan et al., 2008). In chapter ẽfChapter3, we have discussed present state of the art of understanding and knowledge of mitotic events focussing on mammalian cells. Based on the present mechanistic understanding of mitotic systems, first integrative mammalian cell cycle model of regenerating liver is implemented in this chapter.

Materials and methods

Lack of data and experiments on mitotic cells during liver regeneration and also lack of complete understanding of various mechanisms controlling mitosis limits the availability of specific mam-

malian cell type data. Therefore, heuristic and ad-hoc approaches were implemented to model mitotic events. Building on previous work that showed the occurrence of cyclin oscillations in models for the cell cycle in embryos (Tyson, 1991; Goldbeter, 1991; Novak and Tyson, 1993; Sha et al., 2003; Pomerening et al., 2003) and yeast (Chen et al., 2004), and in less detailed or partial models for the mammalian cell cycle (Aguda and Tang, 1999; Qu et al., 2003a,b; Swat et al., 2004; Novak and Tyson, 2004), I present an integrated model of mammalian cell cycle focusing on the cell cycle progression response during liver regeneration. To this end, the control by cell mass was disregarded, which appears less stringent in mammalian cells than in yeast (Conlon and Raff, 2003).

The model is based on the sequential activation of cyclin-dependent kinases. Using the existing knowledge of various cyclin control mechanisms and their temporal organization during liver regeneration, the network controlling the cyclin-dependent kinase activity at various stages of the cell cycle was designed(Fig. 6.1). Using basic principles of biochemical kinetics the model scheme was translated into a set of non-linear ordinary differential equations. Protein degradation was always assumed to follow a linear kinetics. The kinetic parameters (see Appendix 1 for details of equations and parameters) were adjusted so that the model represents the experimentally observed temporal dynamics of each protein species responsible for entry and exit from mitosis. Some of their knock-outs were also simulated. All protein concentrations in the model are expressed in arbitrary units (a.u.) since for most of the regulatory proteins the actual concentrations are not known.

To limit the number of variables in the model, we only considered explicitly the proteins but not the corresponding mRNAs. Thus, induction of gene expression by pro inflammatory cytokines and growth factors and by E2F, were incorporated directly as functions modulating the synthesis of cyclins. Focusing on proteins also allowed us to readily incorporate post-translational regulation through phosphorylation-dephosphorylation or through the formation of complexes with p21/p27. For similar reasons of simplicity I did not distinguish between the nuclear and cytosolic compartments of the cell. The model describes the dynamics of the cyclin-cdk network within one cell.

For numerical simulations of the model Matlab was used and for bistability analysis the computer program XPPAUT with the "stiff" integrator was used.

6.2 The model mechanisms and temporal organization

6.2.1 Mitosis

The regenerating liver has proven to be an excellent *in vivo* model system to study the mechanisms of growth control within a natural tissue environment. Liver regeneration after both

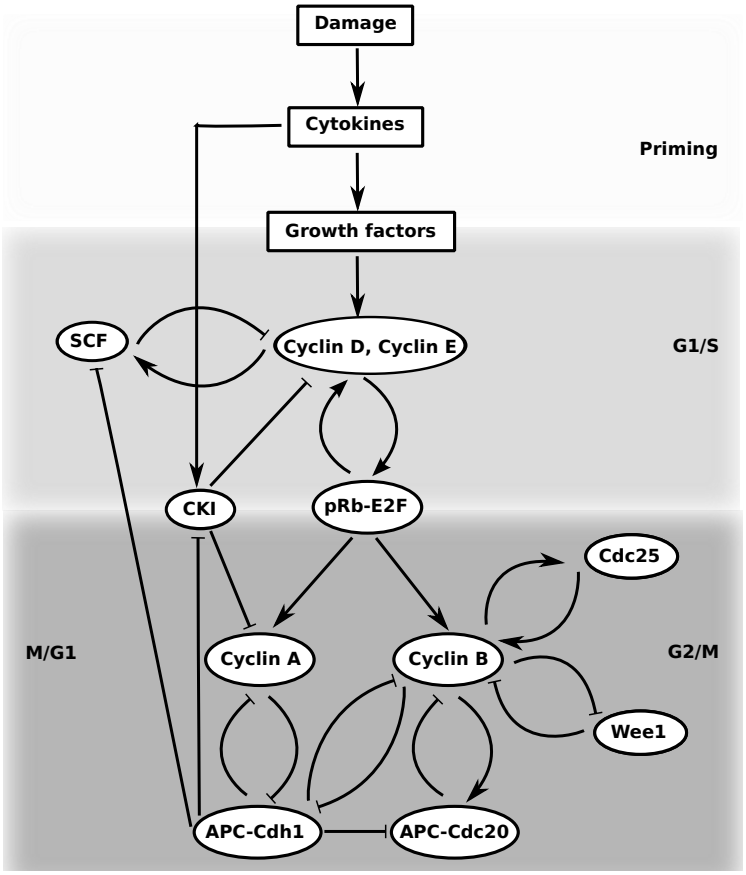

Figure 6.1:
Damage induced model of the cell cycle during liver regeneration based on sequential cyclin activation and degradation. PH induced *Damage* pushes the quiescent cells to *priming* via cytokine signaling. Primed cells then activate growth factor induced signaling cascades. Cytokine- and growth factor induced pathways act in concert to provide mitogenic signals for *Cyclin D* activation in early G1 phase. *Cyclin D* initiates a chain of sequential phosphorylation of Rb-E2F which leads to sequential transcription of further G1-S (*Cyclin E*), S (*Cyclin A*) and M (*Cyclin B*) phase cyclins. Also, stoichiometric inhibitors of cyclins, *CKIs*, are activated by cytokines already at early G1. G1-S proteolytic degradation machinery, *SCF*, is set on by *Cyclin E*. Entry to mitosis is controlled by abrupt Cyclin B-Cdk1 activation at G2-M by *Cdc25* and *Wee1* mediated positive feedbacks. *Cdc25* and *Wee1* mediated positive feedbacks also regulate rapid exit from mitosis by swiftly inactivating Cyclin B-Cdk1 at M/G1 in coordination with positive feedback from M/G1 degradator APC^{Cdh1} and a negative feedback exerted by mitotic degradator APC^{Cdc20}. All cyclin-cdk complexes are depicted as respective activating cyclin partner for simplicity. *E2F* in the scheme represents all three forms of E2F, *viz*, unphosphorylated, hypophosphorylated and hyperphosphorylated.

PH and CCl_4 treatment exhibits well-synchronized DNA synthesis and mitosis (Deguchi et al., 2002).Temporal proliferation pattern of the cell in regenerating rodent liver consists of two waves of mitosis (GRISHAM, 1962; Fabrikant, 1968). During the first round, hepatocytes synchronously undergo DNA replication (peak at 36 hour), and mitosis (peak at 45 hour), which lasts more than 72 hours (Faktor, 1971). Its is then followed by a second round of divisions of lower synchrony. The mitotic peak occurs 4-12 hours after the peak of DNA synthesis at 36 hours (Matsuo et al., 2003a).

6.2.2 E2Fs regulating sequential activation of cyclins

E2F activity is tightly controlled by binding to the retinoblastoma protein (Rb). Rb sequesters E2F and inhibits its transcriptional activity. This hold of Rb on E2F depends on its phosphorylation level. Higher the Rb phosphorylation level more the E2F is released from its hold and is available for transcriptional activation of further downstream genes. Phosphorylation of Rb is regulated sequentially by various cyclin-associated-kinases as discussed in section 3.2. At mid G1 Cyclin D-Cdk4/6 complexes initiate the phosphorylation of Rb. Complete phosphorylation of Rb and E2F release requires further phosphorylation by Cyclin E. Cyclin E hyperphosphorylates Rb during G1-S phase releasing more E2F, which then leads to Cyclin A and Cyclin B transcription(Lundberg and Weinberg, 1998). Also, transcription factors like FoxM1 and B-Myb are known as activators of transcription of mitotic cyclins. FoxM1 is more specifically studied during liver regeneration.

In the model, E2F, FoxM1 and B-Myb are lumped in to a single species called E2F. E2F exists in three forms: unphosphorylated, hypophosphorylated and hyperphosphorylated. Cyclin D initiates Rb phosphorylation and E2F release yielding hypophosphorylated Rb-E2F$_p$. Rb-E2F$_p$ initiates Cyclin E transcription which further phosphorylates Rb-E2F$_p$ giving rise to more E2F and hyperphosphorylated Rb-E2F$_{pp}$. E2F released by hyperphosphorylated Rb-E2F$_{pp}$ is then able to start the initiation of Cyclin A and Cyclin B transcription (Calzone et al., 2008b) (Fig. 3.5). Detailed equations of Rb-E2F phosphorylation dynamics can be seen in Appendix 3.

Cyclin D and Cyclin E mediated phosphorylation/dephosphorylation reactions of Rb and association/dissociation of its 3 phosphorylated forms with E2F are fast enough so that the three phosphorylated forms of Rb and their complexes with E2F are always in equilibrium and hyperphosphorylated form of Rb-E2F can be described as an algebraic equation with the pool of E2F dependent on the temporal evolution of Cyclin D and Cyclin E (Appendix 3). E2F mechanism is closely related to the one considered by Swat et al. (Swat et al., 2004) with some slight modifications.

6.2.3 CKIs at the interphase of S phase and mitosis

CKIs are grouped into two categories: Ink4 and Cip/Kip. Ink4 proteins inhibit Cyclin D associated kinase activity. Cip/Kip family consists of three members: p21, p27 and p57. These proteins act as stoichiometric inhibitors of Cdk2 and Cdk1 (Vidal and Koff, 2000). We lump all forms of CKIs together and refer to them as CKIs unless stated otherwise.

CKIs work as breaks providing additional regulation to the timely cell cycle dependent expression of cyclins. CKIs are predominantly transcriptionally regulated via IL-6 dependent STAT3, Myc and E2F signaling (Gartel and Tyner, 1999; Coller et al., 2000). *CKIs* genes are also reported to be transcriptional cofactors regulating the activity of E2F, STAT3, Myc and, in turn,

the transcription events regulated by them (Coqueret, 2003). Thus, in our model we assume *PICs* which constitutes IL-6, as an inducer of *CKIs* activation.

From the available data on various CKIs proteins we conclude that CKIs are markedly induced after PH, beginning during G1 phase and peaking during the post-replicative phase (48 h). At 120 h after PH, CKIs are again barely detectable (Albrecht et al., 1998). CKIs are degraded by proteolytic degradators. More detailed regulation of CKIs during mitosis is described in section 3.3.

In the model, CKIs exert a two-way control on cyclins. First, CKIs stoichiometrically inhibit Cyclin D, Cyclin E and Cyclin A by forming complexes with their cdk partners. Second, degradators SCF (G1-S controlled) and APC^{Cdh1} (S and mitotic cyclin controlled) drive degradation of CKIs imposing another level of feedback control on cyclins.

6.2.4 Cyclins

The whole process of cell division is mainly orchestrated by cyclin dependent kinases. As the cells progress through the cell cycle, abrupt changes in the enzymatic activities of these kinases lead to changes in phosphorylation state and thus the state of activation of proteins that govern the cell cycle processes. Concentrations of these kinases are constant throughout the cell cycle. Oscillations in their activity depend on the corresponding oscillations in the levels of their respective cyclin subunits. Different cyclins are produced at different cell cycle stages with additional controls imposed on them by various other cell cycle regulators (Fig. 3.3), resulting in a series of cyclin-cdk complexes which govern distinct cell cycle events. Cyclin D and Cyclin E are G1-S cyclins which trigger DNA synthesis as discussed in chapter 2 and 5. The rise of G1-S cyclins is accompanied by the appearance of Cyclin A. Later mitotic transitions are controlled by Cyclin B (Fig. 3.2).

Cyclin A Cyclin A has a function in both S phase and mitosis (Pagano et al., 1992) and it is associated with both Cdk1 and Cdk2 (Garnier et al., 2009). Transcription of Cyclin A is mediated by hyperphosphorylated Rb-E2F (Lundberg and Weinberg, 1998). Cyclin A levels are low during G1 and increase at the onset of S phase, when it contributes to the stimulation of DNA synthesis (Resnitzky et al., 1995). Degradation of Cyclin A at the end of G2 leads to Cdh1 dephosphorylation enabling association of Cdh1 with APC. Active APC^{Cdh1} in turn, leads to timely degradation of Cyclin A (den Elzen and Pines, 2001). Regulation of Cyclin A during mitosis is discussed in section 3.4 in more detail.

Thus in the model, mutually antagonistic control of *E2F* induced *Cyclin A* and APC^{Cdh1} constitutes a positive feedback on Cyclin A degradation and APC^{Cdh1} activation at the end of G2.

Cyclin B Transcription of Cyclin B is mediated by various transcription factors like E2F, NF-Y, FoxM1, and B-Myb. Cyclin B protein forms a complex with Cdk1. Expression of Cyclin B protein in hepatocytes starts in mid S phase. Cdk1 binds to Cyclin B during G2 phase, but the complex is maintained inactive due to inhibitory phosphorylation at Threonin14 (T14) and Tyrosine 15 (Y15) residues by Wee1. This inactive pool of Cyclin B/cdk keeps building during G2 until its inhibitory phosphorylation on T14 is removed abruptly by Cdc25 at G2-M transition. Active Cyclin B in turn activates Cdc25 by phosphorylating it. Active Cyclin B also inactivates Wee1 by phosphorylating it and thus removing the Wee1 imposed Cyclin B inhibition. This abrupt activation and inactivation of its activator Cdc25 and deactivator Wee1, respectively, gives rise to a sudden flood of active Cyclin B which drives mitosis. In the late G2 phase, a very transient induction of Cyclin B-Cdk1 activity is also observed in hepatocytes (Loyer et al., 1994). Thus Cdc25 and Wee1 constitute two feedback loops which get simultaneously activated during G2-M transition. The feedback loops create bistability, such that Cdk1 activation switches from off to on and does not exist in an intermediate state of activation.

At metaphase, sufficiently high levels of active Cyclin B enables phosphorylation of its degradator Cdc20. Phosphorylated Cdc20 activates APC by forming an active complex APC^{Cdc20}, which in turn degrades Cyclin B via proteolysis. This Cyclin B-APC^{Cdc20} system constitutes a negative feedback system controlling the mitotic exit.

When the Cyclin B-Cdk1 complex becomes inactive due to Wee1 mediated phosphorylation and APC^{Cdc20} mediated degradation of Cyclin B, Cdh1 cannot any more be kept in its inactive phosphorylated form. Dephosphorylation of Cdh1 leads to its assembly with APC and the activation of the degradation machinery of APC^{Cdh1}. Moreover, Cdk1 inactivation at mitotic exit leads to Wee1 activation and Cdc25 inactivation due to their dephosphorylation. Thus the mitotic exit system constitutes three positive feedbacks from APC^{Cdh1} , Wee1 and Cdc25 which lead to an irreversible exit from mitosis.

More detail of how Cyclin B activation leads to mitotic entry and its degradation results in exit from mitosis can be found in section 3.4.

6.2.5 Cdc25 and Wee1

Cdc25 A, -B and -C are expressed at different times during the cell cycle. All three coordinate together to dephosphorylate Cdk1 and enable active Cyclin B-Cdk1 complex formation at G2-M transition. In mice hepatocytes Cdc25 B mRNA levels peaks between 40 to 44 hours and a substantial increase in nuclear staining of Cdc25 B protein at 40 hours. Cdc25 A protein shows sustained expression throughout the period of hepatocyte proliferation. For simplicity we lump all forms of Cdc25 into one which is activated during mitosis. Also, the activation of Cdc25 is controlled via phosphorylation by Cyclin B-Cdk1 ((Hoffmann et al., 1993; Izumi et al., 1992; Kumagai and Dunphy, 1992)).

Wee1 mRNA levels in mice hepatocytes are high at the start of cell cycle and during mitotic exit at 72 hour. Wee1 protein levels and its activity in mice hepatocytes are seen to be significantly visible through out the cell cycle with a peak at 72 hour (Murata et al., 2007). Cdc25 and Wee1 mediated control of Cyclin B is explained in detail earlier in this section and in section 3.6. In our model, Cyclin B-Cdk1 is held inactive till G2 phase by Wee1 mediated phosphorylation at two of its inhibitory residues T14 and Y15. Only when T14 phosporylation on Cdk1 is removed by Cdc25 that Cyclin B-Cdk1 becomes fully active. Partial phosphorylations and dephosphorylations by Wee1 and Cdc25 are assumed to be fast enough so that we only have two forms of Cyclin B, fully active and inactive. Phosphorylation of Wee1 by active Cyclin B-Cdk1 renders it inactive during mitosis.

6.2.6 APC and SCF: the degradation oscillators controlling the cell cycle

APC As discussed in section 3.5, APC is the proteasomal machinery degrading proteins after being consecutively activated by two of its subunits Cdc20 or Cdh1, both having a separate window of activity. Cdc20 is activated by Cyclin B-Cdk1 dependent phosphorylation during mitosis. Active Cdc20 binds to APC, which then degrades Cyclin B. Cdh1 is inactivated by Cyclin B-Cdk1 dependent phosphorylation. Thus Cdh1 becomes active at the end of mitosis, when Cyclin B is degraded and assembles with APC to further degrade mitotic cyclins, Cdc20, CKIs and SCF. Cdh1 also acts as G1 regulator destroying mitotic cyclins and CKIs during G1 and maintaining duration of G1 by destruction of SCF (Bashir et al., 2004; Wei et al., 2004). Thus Cdh1 can be considered a master regulator of the cell cycle connecting mitosis to G1-S by degrading G1-S inhibitors like SCF and CKI. It controls Cyclin A and -B at two levels: first in a direct double feedback loop manner and second by inhibiting their inhibitors CKIs and Cdc20 respectively. Cdh1 also controls G1 cyclins, Cyclin D and - E, also by two mechanisms: Firstly by degrading their degradator SCF and secondly by degrading their stoichiometric inhibitor CKIs (Fig. 6.2).

SCF As described in section 2.3 and 3.5, SCF can degrade CKIs and G1 cyclins. SCF is down-regulated in late M and G1 and induced by Cyclin E only when cells near S phase and its degradator Cdh1 is degraded (Bashir et al., 2004). Thus, Cdh1 connects the SCF driven G1-S proteolytic machinery with APC dependent mitotic and G1 proteolysis. Therefore, in the core of cell cycle lies Cdh1 driven degradation which results in an interwoven proteolytic oscillator controlling the cell cycle transitions.

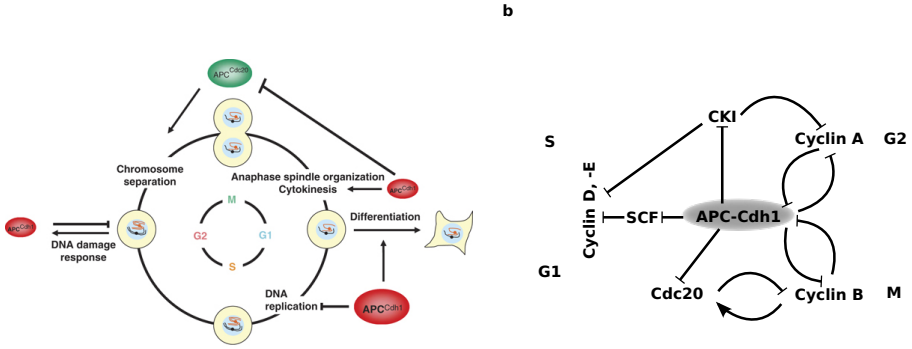

Figure 6.2:
Cdh1 at the core of cell cycle controlling cyclin dynamics. (a) The APC is activated by Cdh1 from the end of mitosis through G1 (APC^{Cdh1}). APC^{Cdh1} controls G1 to either allow differentiation or to prepare for a new round of cell division. In G2, APC^{Cdh1} can be activated in response to DNA damage to block mitotic entry and to initiate DNA repair. In mitosis APC activated by Cdc20 (APC^{Cdc20}) mediates chromosomal separation and initiates mitotic exit. At the end of mitosis APC^{Cdh1} inactivates APC^{Cdc20} and modulates anaphase-spindle dynamics and cytokinesis. Inactivation of Cdh1 can contribute to tumorigenesis by deregulation of these cell-cycle transitions (adapted from (Wasch et al., 2010)). (b) APC^{Cdh1} controls G1-S cyclins by degrading their degradator *SCF* and stoichiometric inhibitor *CKIs*. At the S phase, APC^{Cdh1} exerts a two-layered control on *Cyclin A*, one by mutually antagonist degradation of *Cyclin A* and secondly by degrading its stoichiometric inhibitor *CKIs*. At the mitotic exit, it degrades APC^{Cdc20}, a mitotic degradator of *Cyclin B*. Further, APC^{Cdh1} degrades *Cyclin B* in a mutually antagonist manner. This intricate control exerted by APC^{Cdh1} plays a crucial role in G2 delay imposed due to untimely degradation of G2-M cyclins.

6.3 Results

6.3.1 Simulations of mitosis model

All the model species will be represented in *italics* in the coming discussions. Cyclin D and Cyclin E from the G1-S model (Chauhan et al., 2008) serve as input for the E2F mediated transcription of Cyclin A and Cyclin B in the mitosis model. Step-wise activation of E2F via Cyclin D and Cyclin E leads to sequential activation of cyclins in a cell cycle phase specific manner (Fig. 3.2), also controlled by other regulators (Fig. 3.3). *Cyclin D* appears at late G1, *Cyclin E* at G1-S transition, *Cyclin A* in S phase and *Cyclin B* in late S phase and mitosis (Fig. 6.3).

Active forms of mitotic players *Cyclin B*, *Wee1*, *Cdc25* and *Cdc20* complexed with *APC* exhibit the observed delayed transient induction at mitotic entry (Fig. 3.4). This delayed and transient induction is enabled due to positive feedback from *Cyclin B* inhibitor and activator *Wee1* and *Cdc25* respectively.

Active *Cdh1* forms a complex with *APC* from late mitosis to G1 phase keeping mitotic and G1 cyclins degraded. *SCF* becomes active during G1-S phase leading to degradation of G1-S

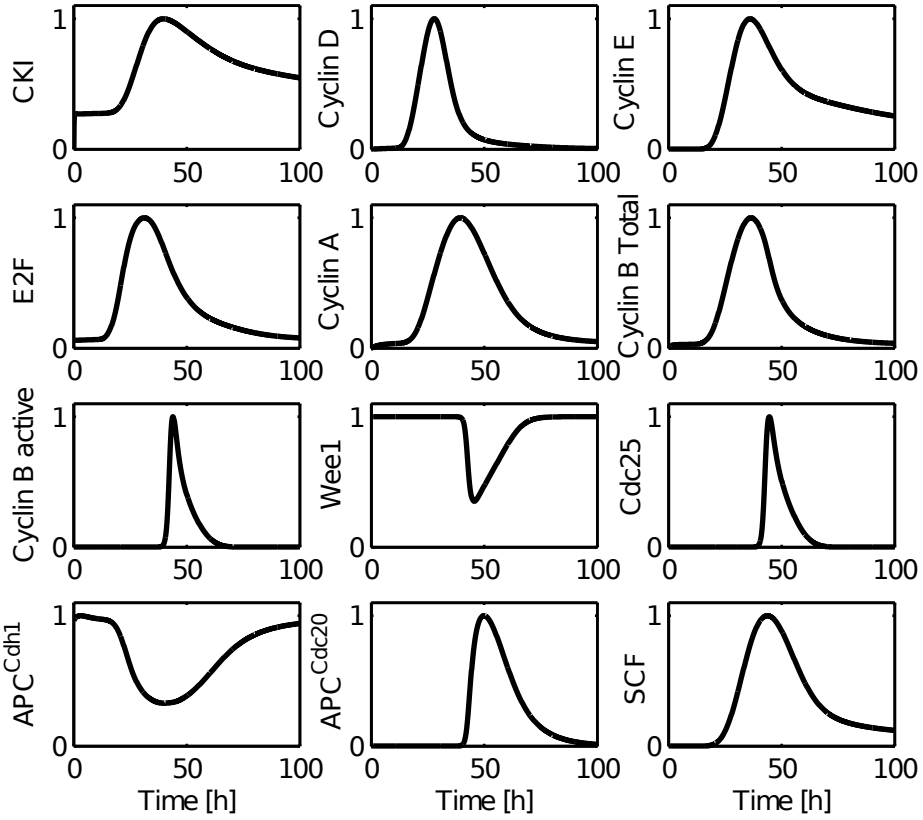

Figure 6.3:
Sequential activation and degradation of cyclins. *Cyclin D* appears at the late G1, *Cyclin E* at the G1-S transition, *Cyclin A* in the S phase and *Cyclin B* in the late S phase and mitosis. At the G2-M interface of mitosis, transient activation of *Cyclin B*, *Cdc25* and inactivation of *Wee1*, leads to abrupt mitotic entry. On the other hand, at the M/G1 interphase of mitosis, *Cdc25* and *Wee1* are respectively rapidly inactivated and activated, leading to swift exit from mitosis. APC^{Cdc20} is also transiently activated once enough *Cyclin B* has accumulated at mitosis. APC^{Cdh1} is active from late mitosis to G1 phase keeping mitotic and G1 cyclins degraded during late mitosis and G1. *SCF* becomes active during G1-S phase leading to degradation of G1-S cyclins. All concentrations are normalized by the maximum concentrations.

cyclins (Fig. 6.3).

Cells in regenerating rodent liver exhibit two waves of mitosis (GRISHAM, 1962; Fabrikant, 1968). During the first round hepatocytes synchronously undergo DNA replication and mitosis. It is then followed by a second round of divisions of lower synchrony. The model is also able to simulate a secondary mitotic peak as observed in experiments (Fig. 6.4) when *Wee1*, *Cdc25* imposed feedback is increased and APC^{Cdc20} dependent negative feedback is delayed (Appendix 5).

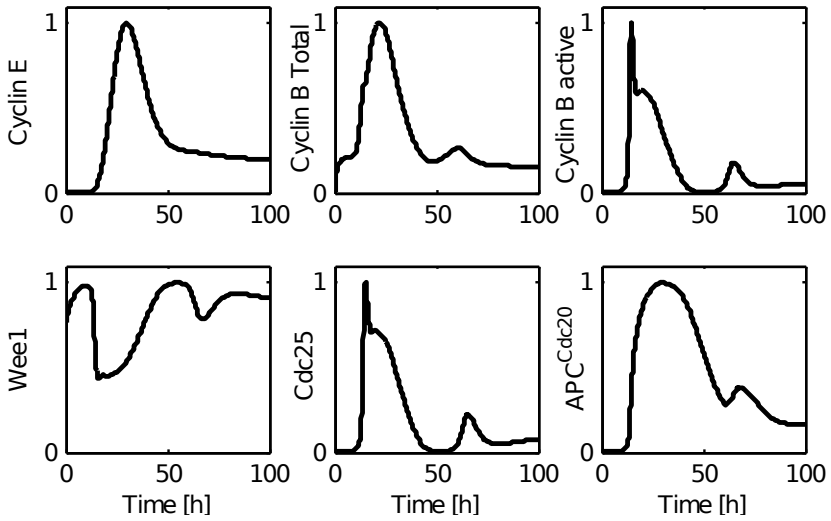

Figure 6.4:
Secondary peak of mitosis as observed in regenerating hepatocytes. Increased positive feedback strength at mitosis and delayed negative feedback at mitotic exit leads to a secondary peak of mitosis. However, Cyclin E levels and so DNA synthesis is unaffected.

Robustness analysis

Liver regeneration is known to be a very robust process, as liver mass reconstitutes itself even in the absence of important cell cycle players. Therefore we expect that the Cyclin E activity, as a measure of total DNA synthesis, to be a robust property of the model. We analyze the peak amplitude and timing of the peak with respect to changes in biochemical parameters over a 100-fold range. Change in Cyclin E amplitude and timing are less than two fold in case of most of the parameters (Fig. 6.5), except the parameters immediately downstream of the input Damage, such as, degradation of *PAI* and *PICs*, *PGE2*, *IEG*. Also, Cycin B is sensitive to changes in these parameters (6.6). This sensitivity is because of the bandpass filtering properties of the model which enables filtering out of very sharp and prolonged damages. Sharp short lived damage input is filtered out due to two incoherent feed-forward loops from *PAI* and *CKI*. On the other hand, weak and long-lived inputs are filtered out due to ultra-sensitivity involved in *HB-EGF* production. Sensitivity to the amount of injury is also well documented in several experiments. Liver regeneration response is proportional to the amount of PH performed. PH of less than 30 % or more than 90 % leads to no liver regeneration response (Lambotte et al., 1997).

Cyclin B shows an all-or-none kind of behavior towards the parameters variations (Fig. 6.6). This all-or-none switch during mitosis is well known and well studied in various experiments and also computational models (O'Farrell, 2001; Csikasz-Nagy et al., 2006), which is due to the bistability observed at the G2-M transition (Fig. 6.17). Once the threshold value of a parameter is reached amplitude and timing of Cyclin B activity is stabilized against any further change in

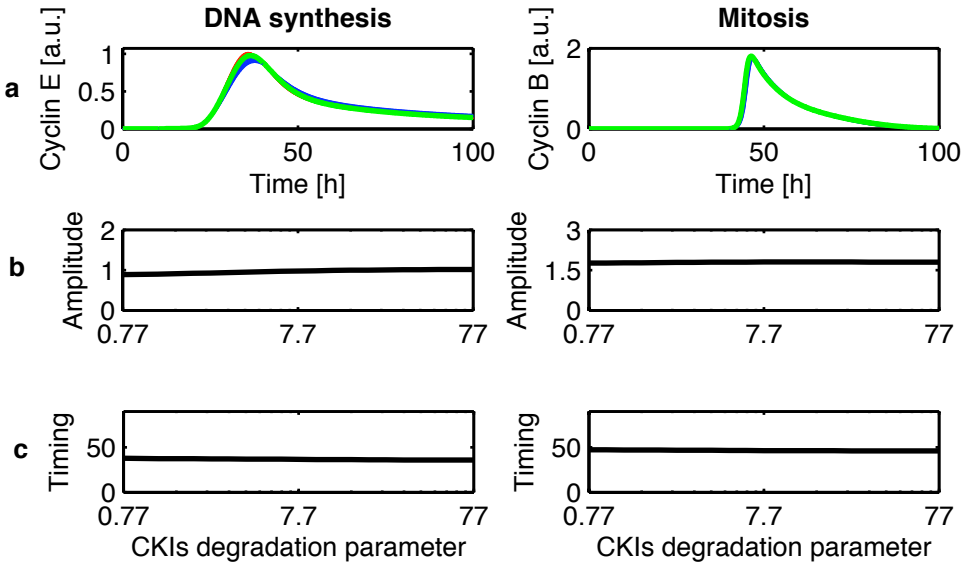

Figure 6.5:
Robustness of the model to parameter variations. Parameter variations at a 100 fold range, transmit only less than two fold change in *Cyclin E*, the indicator of DNA synthesis, and *Cyclin B*, the indicator of mitosis. (a) Thick green lines represent time courses of respective cyclins at the default degradation rate of *CKIs*. Red lines and blue lines indicate time courses with up to 10 fold increase and up to 10 fold decrease respectively, in the parameter values respectively . (b) Peak amplitude of the respective cyclins with respect to fold change in parameter. (c) Timing of the peak amplitude of the respective cyclins with respect to fold change in parameter.

parameter values except for those of *Wee1* production (Fig. 6.8).

Timing of entry of cells into mitosis is known to differ with the time of the day. Wee1 plays an essential role in this circadian control of cell cycle which is illustrated further in our circadian mutant simulations in section 6.3.2 . Simulations of our robustness analysis are consistent with the observation that expression of Wee1 follows a circadian cycle, and the timing of entry of G2 cells into mitosis varies inversely with levels of Wee1 after PH (Fig. 6.8).

A fascinating counterpart to these observations is that the timing of Cyclin E activity and thus DNA replication is insensitive to all the players active during mitosis. This is consistent with the observation that DNA replication is not under the control of circadian rhythms and appears to be an intrinsic property of hepatocytes. Rats and mice differ in the timing of DNA replication after PH, which is 12 to 16 hours earlier in rats. Weglarz and Sandgren transplanted rat hepatocytes into the livers of mice after PH and found that rat hepatocytes replicated earlier than mouse hepatocytes in the resultant chimeric liver (Weglarz and Sandgren, 2000). These results support the fact that the timing of hepatocyte DNA replication after PH is an autonomous process, primarily guided by intrinsic signals.

Parameters linked to *Cdh1* and *CKIs* mostly induce a prolongation or shortening of *Cyclin E*

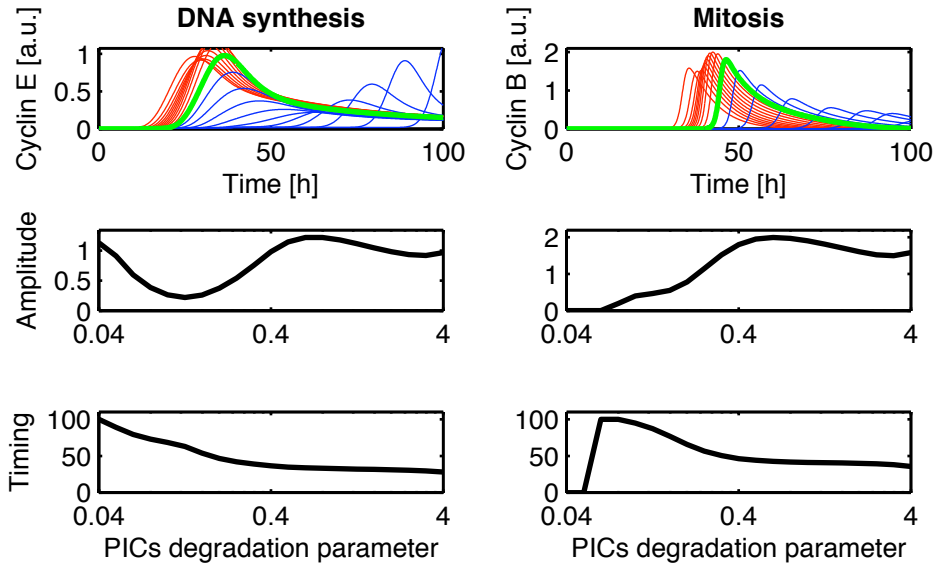

Figure 6.6:
Sensitivity of DNA synthesis and mitosis towards variations in the input. *Cyclin E* and *Cyclin B* are sensitive to variations in the parameters which are directly downstream of the input such as degradation parameters for *PICs*. Amplitude and timing of the peak exhibit bandpass filter type input/output relationship.

degradation. Prolonged degradation corresponds to a delay in emergence of *Cyclin B* (Fig. 6.9). This reflects the delayed mitosis and G2 delay observed in the experiments when DNA synthesis is not completed in time (Park et al., 2007). *CKI* mediated inhibition via Erk signaling is implicated in many of the G2 and mitosis delay studies (Dangi et al., 2006). Cdh1 mediated delay has also been recently reported (Holt et al., 2010). Section 6.3.2 further probes into the the possible role of *Cdh*1 during G2 delay.

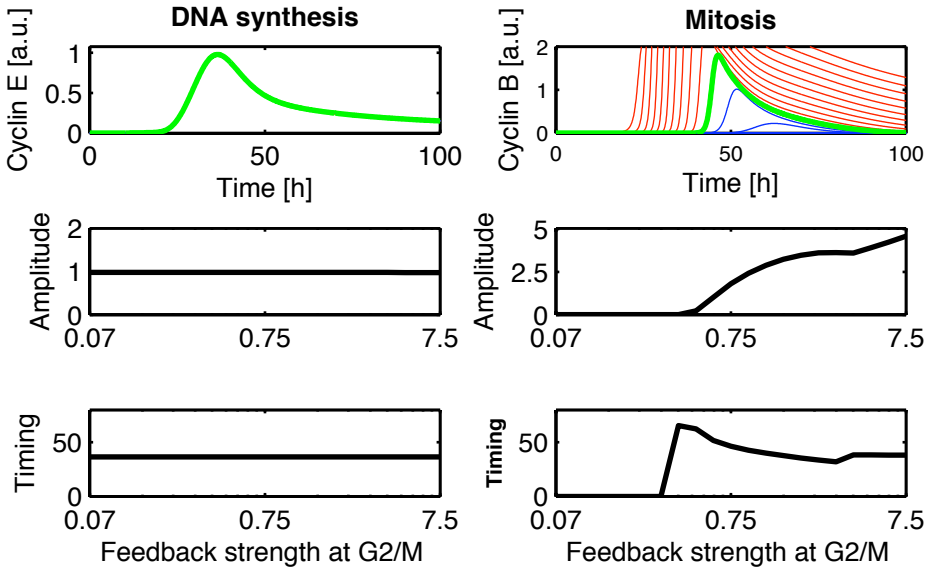

Figure 6.7:
All-or-none behaviour of *Cyclin B* towards parameter variations. Fold change in the parameters, e.g. positive feedback strength parameter, above a threshold value, switches on *Cyclin B* activity abruptly to high levels. Thus *Cyclin B* activation and hence G2-M transition does not jump start as long as the feedback strength exceeds a certain critical value.

6.3.2 Simulations of knockouts

Circadian Clock-Wee1 pathway controlling mitosis

Cell division in many mammalian tissues is associated with specific times of the day. However, how the circadian clock controls the timing of cell division is a hot topic of research due to its direct implications in tumor research and chemotherapy. To explore the relationship between cell division and circadian rhythms, Matsuo et al. (Matsuo et al., 2003b) used a mouse model with partial hepatectomy (PH). PH was performed on mice at ZT8 or ZT0 (ZT0 represents lights on and ZT12, lights off) to compare the kinetics of subsequent cell cycles. The kinetics of S-phase hepatocytes for both ZTs were comparable. In contrast, subsequent mitotic waves at ZT8 or ZT0 differed between 4 to 8 hours(Fig. 6.10). Analysis of the expression profiles of 68 cell cycle-related genes showed only three genes – cyclin B1, Cdc2 (homologue of Cdk1), and Wee1 having remarkably different expression profiles between ZT8 and ZT0 and all three are mitosis regulated (Fig. 6.11). These results suggested that the time of surgery has a marked effect on the timing of mitosis controlling the progression of cell cycling itself.

Wee1 transcription is directly regulated by circadian clock components: activated by Clock-Bmal1 heterodimers and suppressed by Per/Cry proteins. Lack of clock regulated *cryptochromes*

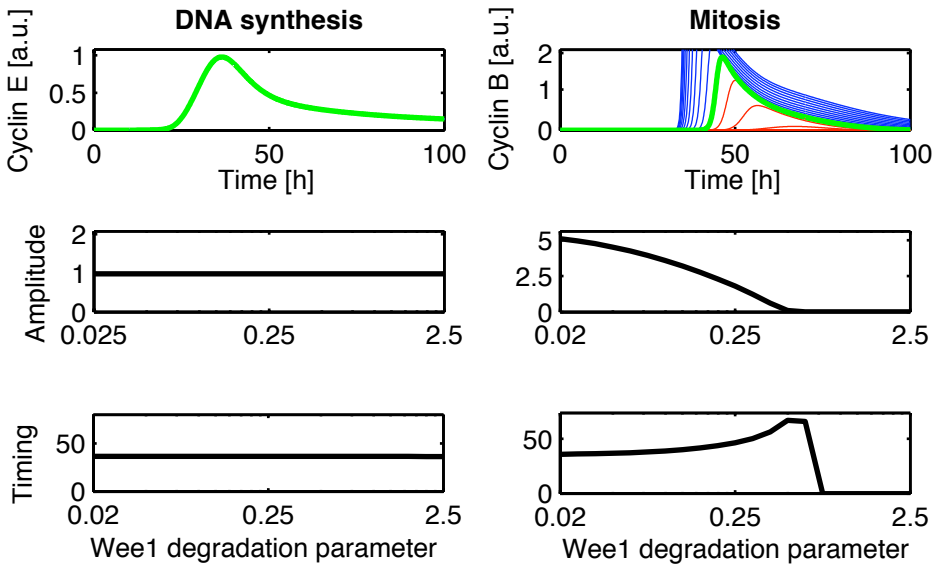

Figure 6.8:
Mitosis is under circadian control of Wee1 while DNA synthesis is autonomous. Fold change in degradation of *Wee1*, which is controlled in a circadian manner, has a strong linear correlation to the timing of *Cyclin B* activity while has no affect on Cyclin E activity. Thus, circadian shifts in Wee1 might control entry of the cells to mitosis, while DNA synthesis is unaffected by external stimuli.

(Crys) causes circadian disruption and impairment of hepatocyte proliferation. Also, Wee1 gene is reported to have E-box elements which are targeted by Clock-Bmal1 heterodimers. In mutated E-box region of Wee1 gene circadian transcription is reduced. In Cry-deficient mice, Wee1 mRNA levels are reported high at both ZTs. However, in Clock mutant (Clock/Clock) mice, Wee1 expression was low at both ZTs. These findings suggest a direct regulation of circadian clock by Wee1 (Matsuo et al., 2003b).

The peak of Wee1 activity in ZT8 occurs 4 to 8 hours before the corresponding mitotic peak in ZT0. In Cry-deficient mice as well Wee1 activity peaks 4 hours before mitotic peak in ZT0. Also, Cyclin B activity occurs 4 hours before in both Cry-deficient and ZT8 mice due to early and increased levels of Wee1. Thus, we use Wee1 as a marker of circadian shift at ZT8 in our model. We reduce degradation of Wee1 to mimic early increased peak of Wee1 in response to PH at ZT8. The model is able to simulate the early peak in Wee1 and mitosis observed in ZT8 mice (Fig. 6.12). Therefore, our model is able to simulate circadian control of cell cycle during liver regeneration.

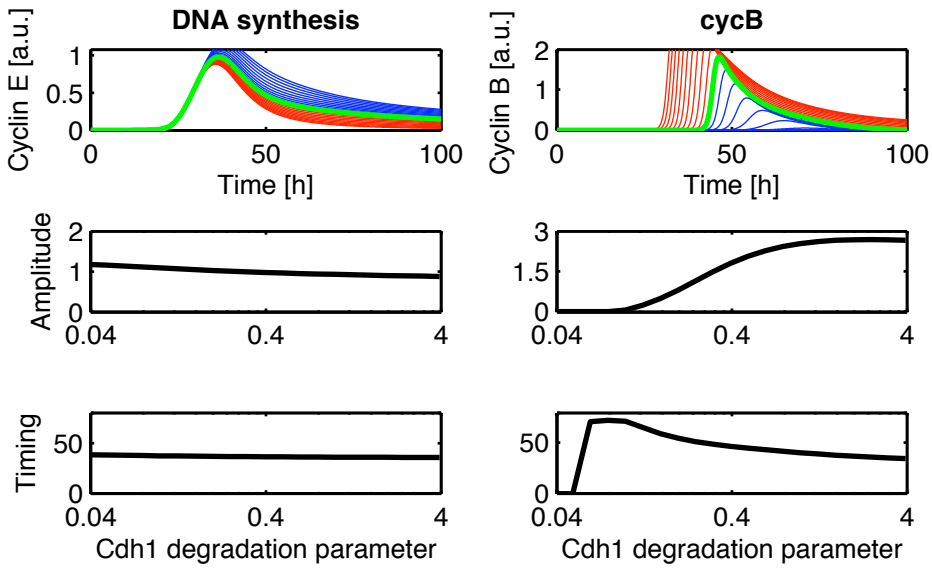

Figure 6.9:
Parameter changes causing G2 delay. Variations in parameters linked to Cdh1 and CKIs result in prolonged *Cyclin E* degradation. This prolongation is directly proportional to the delay in the timing of *Cyclin B* activity and hence mitosis. Thus, *Cdh1* and *CKI* might have a role to play in untimely degradation of cyclins leading to G2 delay.

Impaired regeneration of fatty liver and mitotic delay

Fatty liver (hepatic steatosis) is the most common hepatic disorder which can be caused by various etiologies such as obesity, diabetes mellitus and alcohol consumption. Impaired liver regeneration in fatty livers is a cause of concern in living donor liver transplantations. Various previous studies have reported some possible causes of impaired liver regeneration in fatty liver after PH including disruption in cytokine signaling and mitotic factors. Murata et al. (2007) studied the mitotic response after PH in fatty liver mice. Mitosis was drastically delayed in fatty liver mice suggesting mitosis to be main cause of proliferation defects in fatty liver mice. The protein levels of Cyclin B between livers in the lean and fatty mice did not substantially differ. Interestingly, Cdc2 which is one of the markers of G2 stage and is the activating partner of Cyclin B, was less phosphorylated in the fatty liver than in the lean group even though Cdc2 protein levels in both groups was almost the same. mRNA and protein levels of Wee1 which phosphorylates Cdc2 at G2 were observed to be reduced and delayed in fatty liver. Thus delayed and reduced expression of Wee1 causes Cdc2 phosphorylation at an inappropriate time leading to delayed appearance of active Cyclin B-Cdc2 complex. In the model we mimicked reduced Wee1 by increasing degradation parameter of Wee1 (see Appendix 5) and the model is able to reproduce delayed and reduced expression of Wee1 and active Cyclin B (Fig. 6.13). Delayed Cyclin B-Cdc2 leads to delayed mitosis and consequently impaired liver regeneration. We simulate the decreased Wee1 observed in fatty liver by increasing Wee1 degradation rate. Thus, our model successfully simulates reduced Wee1 as a cause of delayed Cyclin B activity causing

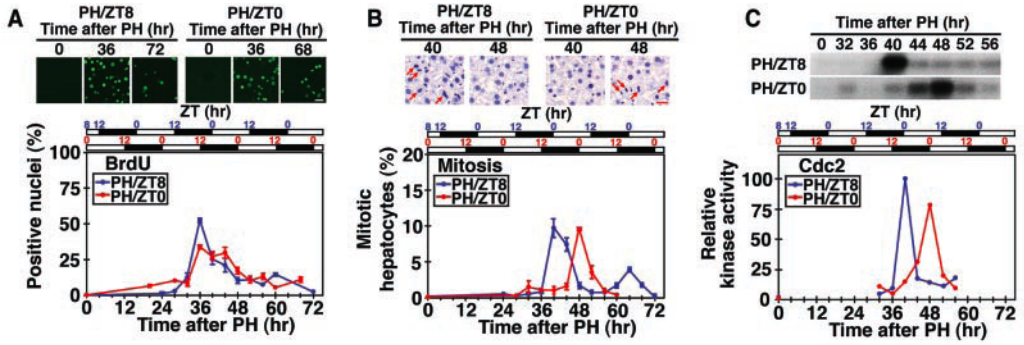

Figure 6.10:
Effects of time of PH on subsequent liver regeneration. Zeitgeber time (ZT) which is a 12 hour constant light- 12 hour constant dark cycle, was used to control the day-night light time. White and black bars above the graphs represent times when lights were on or off, respectively. (A) Kinetics of DNA synthesis (BrdU-incorporation) in hepatocytes does not differ much between PH performed at ZT8 and PH performed at ZT0. (B) Kinetics of mitotic hepatocytes after PH at ZT8 is 4-8 hours earlier than PH at ZT0. (C) Kinetics of Cdc2 kinase activity after PH at ZT8 is 4-8 hours earlier than PH at ZT0 (Matsuo et al., 2003b).

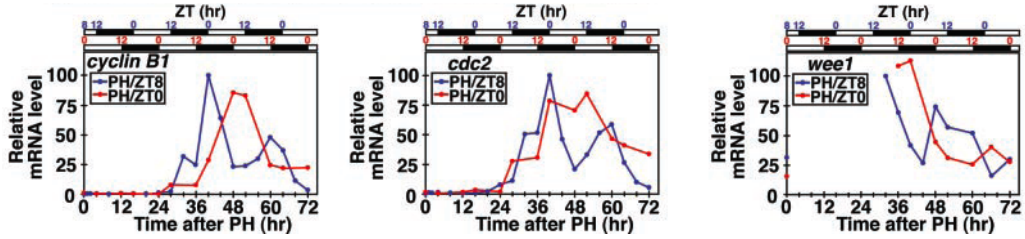

Figure 6.11:
Temporal expression profiles of circadian clock regulated cell cycle genes. 3 cell cycle regulated genes, Cyclin B1, Cdc2, and Wee1, showed remarkably different expression profiles between PH at ZT8 and PH at ZT0 (Matsuo et al., 2003b).

impaired liver regeneration in fatty liver mice.

Interestingly, fatty liver disorder is also subjected to circadian regulation. Circadian mutants are more susceptible to fatty liver disorder (Turek et al., 2005; Kudo et al., 2009). Wee1 is one of the mediators of reduced mitosis observed in impaired regeneration of fatty liver mice. Wee1 is also the gatekeeper of circadian regulation of cell cycle as already discussed above. Fatty liver disorder being regulated by circadian rhythms, and Wee1 being important for both circadian regulation and fatty liver disorder, suggests Wee1 to be a very important knockout model to study fatty liver disorders.

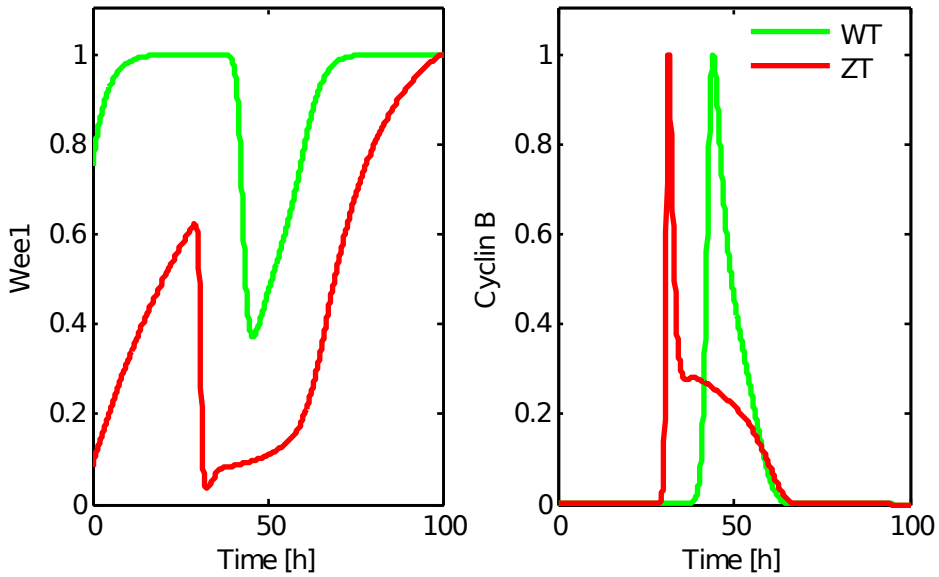

Figure 6.12:
Cyclin B and *Wee1* activity in response to PH performed at ZT0 and ZT8. WT in the figure represents the PH performed at ZT0 and ZT represents PH performed at ZT8, when *Wee1* and *Cyclin B* activity peak earlier as observed in Fig. 6.11. Note that the normalized amplitudes are plotted here since the focus is on delay.

FoxM1 in proliferation and G2 arrest

FoxM1 is a cell cycle regulated gene with peak expression during G2-M (White et al., 2005). *FoxM1* is a typical proliferation associated transcription factor which is in particular re-expressed in liver regenerative proliferation following injury. Liver regeneration studies with genetically altered mice that either prematurely express *FoxM1* in hepatocytes (Ye et al., 1999) or contain a hepatocyte-specific deletion of the FoxM1 targeted allele (Wang et al., 2002) demonstrate that *FoxM1* is required for hepatocyte proliferation through the control of the levels of cell cycle regulatory proteins. It is found to be over expressed in majority of human tumors. *FoxM1* knock-out models in mice indicate that it contributes to both tumor initiation and progression.

FoxM1 is also implicated in transcriptional control of many genes involved during G2-M transition including Cyclin A, Cyclin B. FoxM1 is active during DNA damage induced G2 arrest. Alvarez-Fernandez et al. (2010) for the first time show that FoxM1 and some residual cdk activity is required for the checkpoint recovery after DNA damage; challenging the traditional view that cdk activity should be completely inhibited during DNA damage to allow for repair. It is not hard to imagine that some aspect cdk controlled cell cycle machinery remains poised for action during DNA damage induced checkpoint. However, with that residual activity of cdk how the cell makes sure that it does not roll into mitosis before that damage is fixed? Interestingly, Alvarez-Fernandez et al. (2010) also show that siRNA mediated depletion of Cyclin A but not Cyclin B strongly inhibits FoxM1 transcription. So one answer to this question would be that

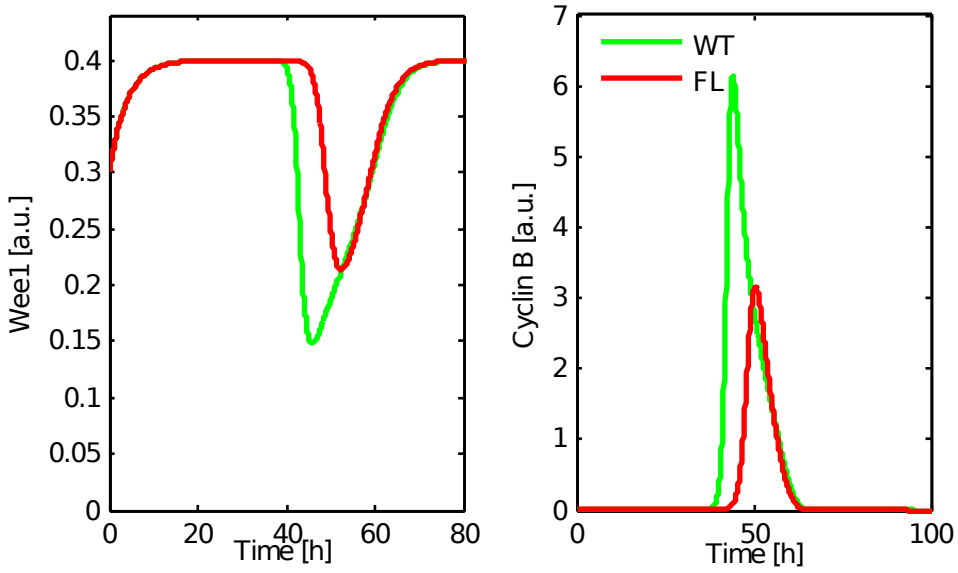

Figure 6.13:
Delayed mitosis in fatty liver disorder due to Wee1 mediated delayed Cyclin B induction. WT is the wild type mice in the figure and FL is fatty liver mice. Both *Wee1* and *Cyclin B* activity is delayed as observed in the experiments (Murata et al., 2007)

.

Cyclin A bound cdk has a residual activity while Cyclin B bound cdk that is essential for mitotic entry, is more completely blocked. In the model, parameters for Cyclin A and Cyclin B dependent transcription were respectively reduced in order to mimic Cyclin A and Cyclin B siRNA (see Appendix 5). The *E2F* species in the model which lumps *FoxM1* is able to exhibit that control of FoxM1 activity is dependent on Cyclin A whereas Cyclin B is dispensable. However, over-simplification of *FoxM1* regulation in our model by lumping it with *E2F*, limits us to further dissect into possible systems level controls.

The new role of FoxM1 in G2-M checkpoint recovery and its intricate control by mitotic cyclins during DNA damage recover makes it an interesting candidate gene for studying G2 arrest.

HGF treatment at S phase causes G2 delay

Growth factors are well known to promote the transition from G1 to S phase in cell cycle progression. Treatment of cells with HGF at S phase induces G2 delay. Mitosis entry is 1-2 hours later with delay starting at G2 (Park et al., 2007). It is well known that the protein levels of Cyclin A and Cyclin B decrease during M phase progression resulting in the decrease of respective kinase activities (Clute and Pines, 1999). G2 delay leads to delay in the degradation of Cyclin A and Cycin B proteins. HGF injection at S phase was mimicked in the model (see Appendix 5)

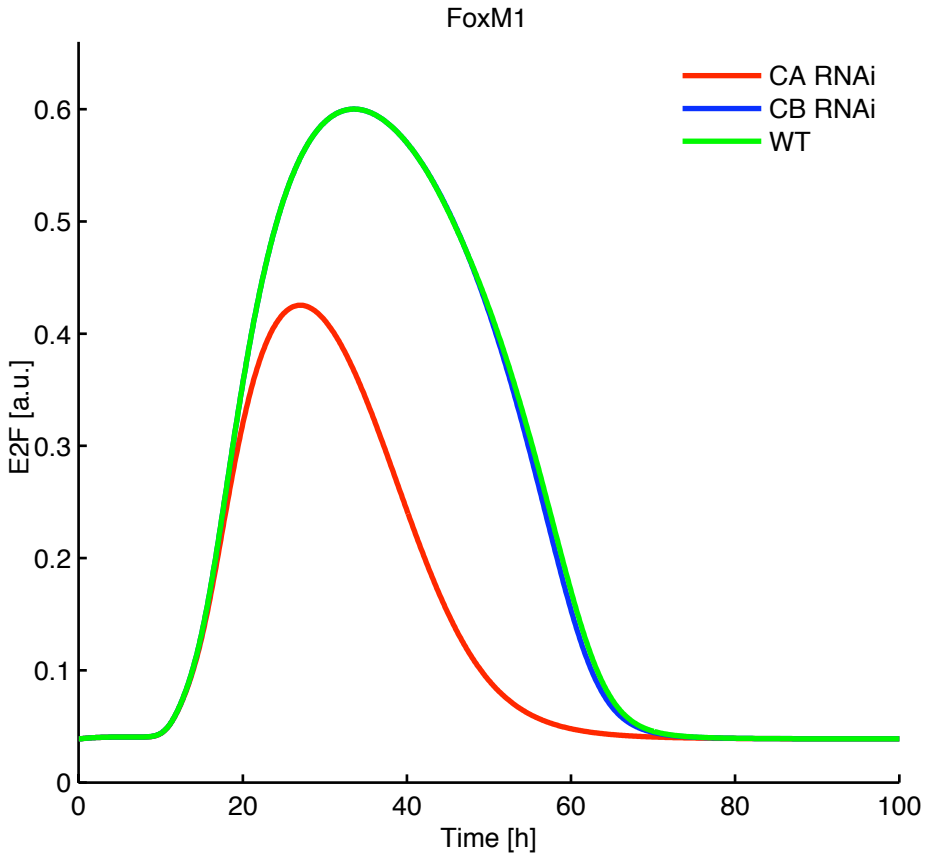

Figure 6.14:
FoxM1 (*E2F* in the model) activity at S-G2 is Cyclin A dependent. *FoxM1* is a typical proliferation associated gene controlling G2-M cyclins during liver regeneration following injury. It is implicated in DNA damage-induced G2 arrest. It is mainly regulated by Cyclin A during S phase, since Cyclin A RNAi significantly reduces its expression while Cyclin B RNAi has no effect (Alvarez-Fernandez et al., 2010). Our model reproduces these Cyclin A and Cyclin B RNAi mutants.

and the model was able to reproduce delayed degradation of Cyclin A and Cyclin B (Fig. 6.15). Erk signaling is known to mediate this G2 delay via CKIs (Han et al., 2005; Dangi et al., 2006; Park et al., 2007). However, our simulations suggest a contributed role of *CKIs*, APC^{Cdh1} and *FoxM1* (*E2F* in the model) dependent delay in *Cyclin A* and *Cyclin B* degradation (Fig. 6.15).

FoxM1 plays a role in recovery from DNA damage induced G2 arrest as already discussed in the previous FoxM1 mutant (Alvarez-Fernandez et al., 2010). Also, the deletion of subunit APC2, leads to metaphase arrest and stabilization of APC substrates such a Cyclin A and Cycin B which may be caused by the lack of APC^{Cdh1} (Wirth et al., 2004; Teodoro et al., 2004). Also, spatial regulation of APC^{Cdh1} mediated Cyclin B degradation has been newly reported to play a role in maintaining G2 arrest (Holt et al., 2010). All these observations suggest a much complex

Cdh1 dependent mechanism controlling degradation of Cyclin A and Cyclin B controlling the G2 arrest in response to growth factor induction at S phase.

As cyclins are major biological targets of APC^{Cdh1}-promoted ubiquitination and degradation, and at the same time cyclin-cdk phosphorylation blocks APC^{Cdh1} activation, creating a positive feedback between cyclins and APC^{Cdh1} . Such circuitry could result in hysteresis with stability of both the G1 low-cyclin/high-Cdh1 state and the S/G2-M high-cyclin/low-Cdh1 state. In our model, APC^{Cdh1} exerts multiple layers of feedbacks on G2-M cyclins. It would be very interesting to study the positive feedbacks exerted by APC^{Cdh1} on *Cyclin A* and *Cyclin B* during G2 delay. Also, in the wake of upcoming experimental evidences of *FoxM1* and APC^{Cdh1} related G2 delay, it would be very interesting to dissect a reduced G2-M model at systems level to understand *FoxM1* control of G2 transcription and its interplay with APC^{Cdh1} to control G2 delay.

6.3.3 Irreversibility of mitotic exit

Cyclin proteolysis, is known to be responsible for the mitotic exit and the systems-level feedback that affects synthesis and degradation rates, make M-G1 transition irreversible. It has been reported that forced cyclin destruction in mitotic budding yeast cells efficiently drives mitotic exit events. However, these remain reversible after termination of cyclin proteolysis, with recovery of the mitotic state and cyclin levels. Mitotic exit becomes irreversible only after longer periods of cyclin degradation, owing to activation of a double-negative feedback loop involving the cdk inhibitor Sic1 (CKIs in mammals) (Kapuy et al., 2009). Quantitative modeling suggests that feedback is required to maintain low cdk activity and to prevent cyclin re-synthesis. Recently, Potapova et al. (Potapova et al., 2009) have reported such an irreversible behaviour of exit from mitosis in *Xenopus* and Hela cells and argued a Wee1/Cdc25 mediated feedback mechanism responsible for irreversibility of mitotic exit after longer periods of cyclin degradation. Thus Wee1 and Cdc25 mediated positive feedbacks at M-G1 transition were dissected. In this regard, a reduced version of our G2-M-G1 model was introduced, conserving all the feedback properties on Cyclin B during G2-M-G1 (Appendix 6).

With this reduced model, we are able to demonstrate bistability observed in the *Cyclin B* production during G2-M transition (Fig. 6.17). Total *Cyclin B* levels are dependent on its production and degradation terms. Its activity is enhanced by positive feedbacks from Cdc25 and Wee1 and its degradation is carried out by APC^{Cdh1} in a positive feedback manner and by APC^{Cdc20} in a negative feedback manner (Fig 6.16). *Cyclin B* nullcline, the S-shaped curve in the plot, is plotted against its production rate, which is the X-axis. Two thick lines represent its two stable steady states and the dashed line represents the unsteady state. At lower stimulus strength *Cyclin B* system always goes back to lower steady state, represented by down arrows. Once the stimulus is increased beyond a threshold value (4.5 on X axis), system switches to upper steady state in an all-or-none fashion, represented by up arrows. Once stimulated to higher level, the system stays at the higher steady state even if the stimulus is decreased below the threshold value required to

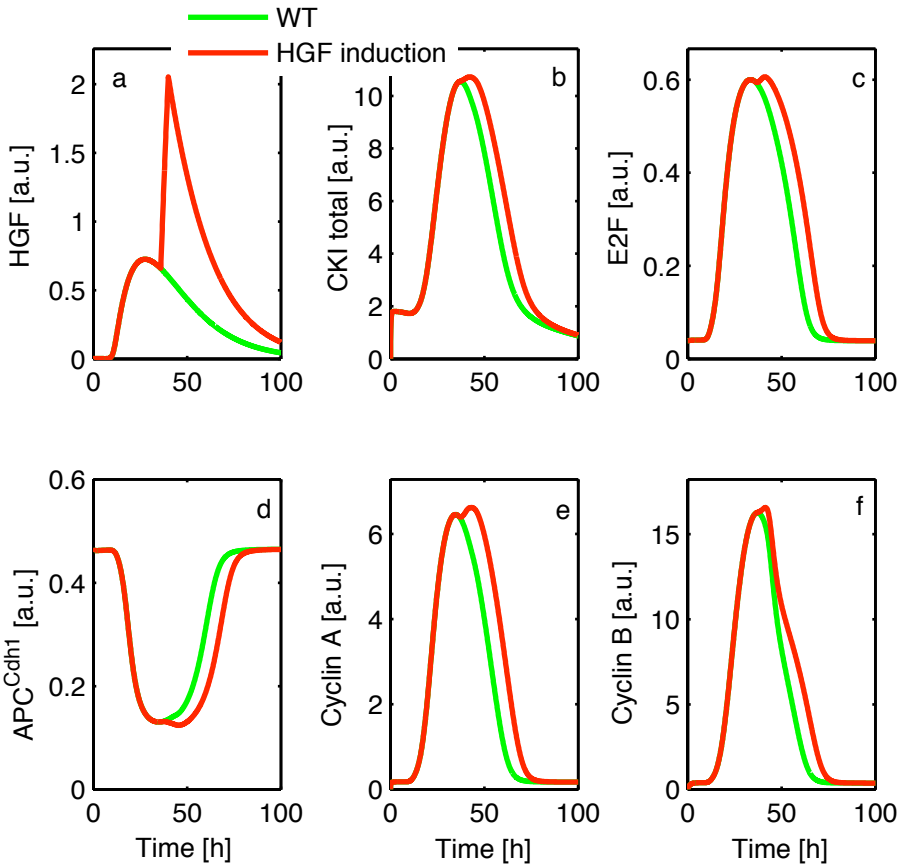

Figure 6.15:
Growth factor induction at S phase delays mitosis. At S phase *HGF* is induced (a) which leads to delay in the degradation of *Cyclin A* (e) and *Cyclin B* (f). *CKI* (b), *E2F* (*FoxM1*) (c) and APC^{Cdh1} (d) contribute together in causing this delayed degradation of G2-M cyclins. Growth factor induction at S phase might lead to increased *FoxM1* (*E2F*) resulting in increased Cyclin A activity. Since two antagonists *Cyclin A* and APC^{Cdh1} can not coexist, APC^{Cdh1} is delayed which leads to delay in *Cyclin A* and *Cyclin B* degradation. Also, delayed APC^{Cdh1} might affect *CKIs* dependent G2 delay.

flip it on.

In order to study irreversibility at mitotic exit, we are studying the dynamics of *Cyclin B* with respect to its *Wee1* mediated positive feedback, which gets activated at G1, and APC^{Cdh1} mediated positive feedback which degrades Cyclin B during late mitosis and G1. It is out of the scope to be part of this thesis, but it would be interesting extension of this thesis to study the role of the feedbacks at M-G1 transition causing irreversibility.

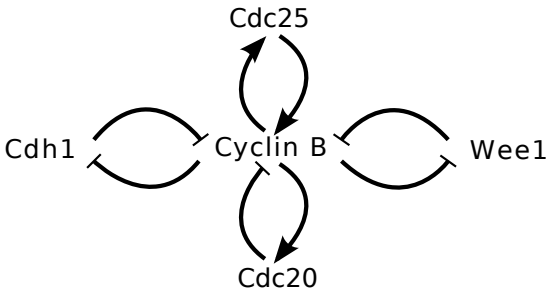

Figure 6.16:
Systems level feedbacks acting on Cyclin B regulation during M-G1. Mitotic exit system constitutes three positive feedbacks from APC^{Cdh1}, *Wee1* and *Cdc25* which lead to an irreversible exit from mitosis.

6.4 Discussion

First damage induced, cytokine and growth factor mediated model of mammalian cell cycle progression is presented in this chapter, focussing on cell cycle events in regenerating livers of rodents. The model couples different cyclin-cdk complexes that control the successive phases of the mammalian cell cycle, which would be capable of describing their sequential activation. The model presents re-entry of normally quiescent liver cells in to cell cycle after being induced by injury. The model illustrates many interesting properties of the mammalian cell cycle, mainly emphasizing on the emerging role of Cdh1 in mammalian cell cycle and of Wee1 during mitotic exit. The role of Cdh1 and *FoxM1* in G2 delay is investigated. The model also reinforces the role of Wee1 as a gatekeeper of circadian regulated cell cycle and also illustrates reported deregulation of Wee1 in circadian regulated diseases like fatty liver.

In the wake of upcoming evidences on the role of Cdh1 and *FoxM1* during DNA damage induced checkpoint recovery, it would be interesting to study various feedbacks in the model imposed by Cdh1 on G2-M cyclins during the control of G2 arrest. Also the model proposes that Wee1/Cdc25 mediated positive feedbacks on Cyclin B at G1 render mitotic exit irreversible.

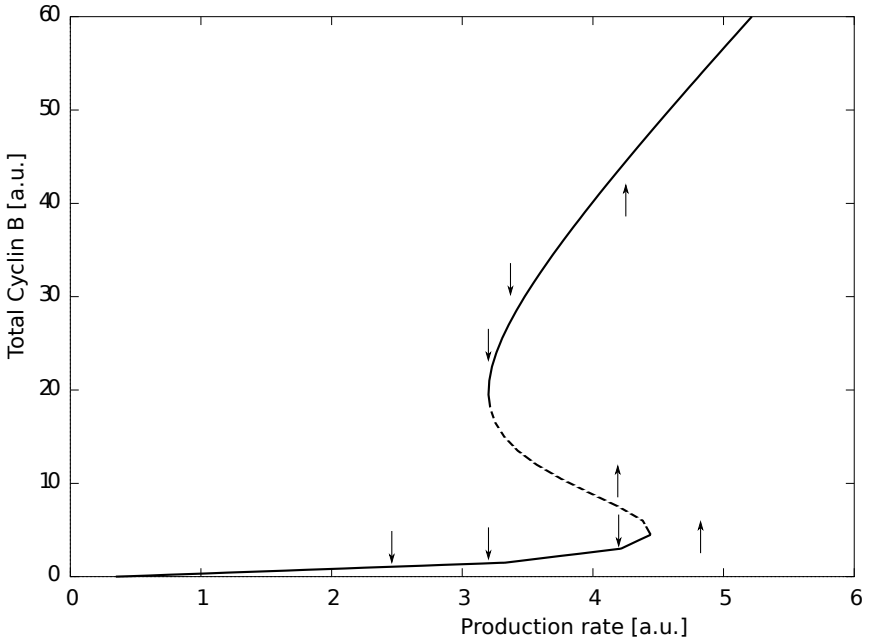

Figure 6.17:
Cyclin B synthesis at G2-M is bistable. Total Cyclin B is dependent on its production (Prod) and degradation via APC^{Cdh1} and APC^{Cdc20}. *Cyclin B* activity is enhanced by positive feedbacks from *Cdc25* and *Wee1* (Fig 6.16). Total *Cyclin B* nullcline is plotted against its production rate. Two thick lines represent two stable steady states of *Cyclin B* and the dashed line represents the unsteady state. At lower stimulus strength *Cyclin B* system always goes back to lower steady state, represented by down arrows. Once the stimulus is increased beyond a threshold value (4.5 on X axis), system switches to upper steady state in an all-or-none fashion, represented by up arrows. Once stimulated to higher level, the system stays at the higher steady state even if the stimulus is decreased below the threshold value which was required to flip it on.

7 Conclusions and outlook

First damage induced model of mammalian cell cycle is presented. The model focuses on the cell cycle during liver regeneration in rodents and illustrates many properties of mammalian cell cycle, including the newly emerging scope and understanding of the ubiquitination machinery in the mammalian cell cycle control system.

Role of Cdh1 in G2 delay

Cdh1 lies at the core of cell cycle control system in our model, connecting mitosis to G1 and having a role to play in G2 arrest. Cdh1 has long been known to be a subunit of the APC dependent ubiquitination machinery active during late mitosis and G1. Five years ago, discovery of the role of Cdh1 in degrading G1/S degradator SCF (Bashir et al., 2004; Wei et al., 2004) and stabilizing p27 in Hela cells opened the window for deciphering the involvement of Cdh1 in maintaining the G1 phase and thus quiescence in mammalian cells. Preserving these Cdh1 dependent properties of G1 control, I went on to further clarify the role of Cdh1 in G2 phase delay.

Traditional understanding of G2 delay involves CKIs. Lately, Cdh1 has also been reported to cause G2 arrest by affecting degradation of Cyclin A and Cyclin B. More specifically, Cdh1 was recently reported to be nuclear guardian of G2 arrest in mice oocytes by controlling degradation of nuclear Cyclin B (Holt et al., 2010). The model simulates both traditional CKIs dependent and recently discovered Cdh1 dependent delay in the G2 phase. Cdh1 helps maintain certain threshold levels of Cyclin B at G2, as suggested by (Holt et al., 2010), which are sufficient enough to cause G2 arrest but not enough to suppress sudden pool of Cyclin B at G2/M transition. It would be very interesting to study the mechanisms that could possibly help maintain such thresholds.

Irreversibility of mitosis in mammalian cells

Exit from mitosis is an irreversible event of the cell cycle. Regulated degradation of mitotic cyclins is a major factor controlling the mitotic exit, but experimental studies have shown that

it only provides directionality to the exit from mitosis (Potapova et al., 2006). There are other mechanisms at the M-G1 that render exit from mitosis irreversible. In yeast cells this irreversibility is provided by the positive feedback cyclin-cdks and CKIs, which is active during M/G1 transition (Tyson and Novak, 2008). Recently, it has been reported that the kinases (Wee1 and Myt1) and phosphatases (Cdc25) that control Cyclin B-Cdk1 activation during G2/M transition also control the low Cdk1 activity during G1. Thus the incidence of irreversibility might be explained with the positive feedbacks induced by Wee1 activation and Cdc25 inactivation in the G1 phase. The model, very clearly shows strong control of Cdc25 and Wee1 in Cyclin B-Cdk1 inactivation in the early G1 phase. We intend to study this feedback system at the M-G1 interphase and understand if this irreversibility in the mitotic exit is caused by the Wee1/Cdc25 induced double positive feedback. To this end, a reduced model system for the mitotic exit was generated. Wee1/Cdc25 mediated positive feedback loops on Cyclin B lead to a bistable switch like activation of Cyclin B, resulting in abrupt G2/M transition.Further the goal is to study how the Cyclin B concentrations evolve with respect to APC^{Cdh1}, the M-G1 degradator of Cyclin B. The bifurcation analysis of this M-G1 system to study the possibility of irreversibility would be an interesting extension of this thesis.

Heterogeneity of cell division in the liver

Although the liver tissue has a uniform histological appearance, it is heterogeneous at the level of morphology and histochemistry. This heterogeneity is linked to the position of a cell within the functional unit of the tissue, which in turn, is related to the blood supply: Cells located in the upstream zone (periportal zone) differ from those in the downstream zone (pericentral zone) with respect to subcellular structures, key enzymes, translocators, and receptors; and therefore, have different metabolic capacities. Also, some hepatocytes divide only once during liver regeneration, while others pass through two or more division cycles. Two models (which are not mutually exclusive) may account for such hepatocyte heterogeneity: (a) individual hepatocytes may significantly differ in their sensitivity towards cytokine concentrations due to random fluctuations in protein expression ('stochastic model'); (b) a cytokine gradient may be formed during liver regeneration, so that hepatocytes nearby injured sites or in the periportal zone of lobules are subject to stronger stimulation, and thereby divide more often ('spatial model').

Discoveries regarding the functional heterogeneity of the various liver cell types, including hepatocytes, hepatic stellate cells, sinusoidal, endothelial, and Kupffer cells, are providing new insights into our understanding of the development, prevention and treatment of liver disease. For example, functional differences along zonal patterns have been demonstrated for the hepatocytes and can explain the gradients and manifestations of disease observed within lobules. Intralobular gradients of many metabolites like glucose uptake, glycogen depletion, xenobiotic metabolism, have been demonstrated.

Extensive simulation studies with populations of proliferating hepatocytes will help generat-

ing predictions that will allow experiments to distinguish stochastic and spatial heterogeneities. Awareness of the complexities and heterogeneity of the liver will add to a greater understanding of liver function and disease processes that lead to toxicity, cancer, and other diseases.

Circadian regulation of cell cycle

Cell division in many mammalian cells is associated with specific times of the day. There is evidence that the cell division cycle is controlled by the intrinsic circadian clock of the cells. Effect of circadian timing of treatment on regenerating liver cells in mice has been systematically carried out by Matsuo et al. (Matsuo et al., 2003b). They investigate Wee1 as the gate-keeper of circadian regulated cell cycle. Our model is able to reproduce their circadian mutants and we reinforce role of Wee1 as a gatekeeper of circadian regulated cell cycle.

Interestingly, fatty liver, which is the most common liver disorder is also subjected to the circadian regulation. Wee1 deregulation is one of the causes of impaired mitosis in fatty liver (Murata et al., 2007). The model simulates Wee1 dependent disrupted Cyclin B activity and thus impaired mitosis in fatty liver. Pertaining to its important role in circadian control of cell cycle and also in regulating circadian diseases, we suggest Wee1 mutant to be an important model system to study circadian regulated tumors and xenobiotic metabolism.

It would be also very interesting to study dynamic simulations of circadian modulations of liver regeneration through oscillating expression of Wee1.

FoxM1 and Cdh1 mutants for mammalian cells

FoxM1 is a typical mitotic transcription factor that is re-expressed in liver regenerative proliferation following injury and is over-expressed in most of the human tumors. It is very widely studied in age related and mitotic defects in regenerating mice livers. Also, recently it has been implicated in G2 delay (Alvarez-Fernandez et al., 2010). We could successfully simulate Cyclin A dependent control of FoxM1 at G2.

Although over-simplification of *FoxM1* regulation in our model by lumping it with *E2F*, limits us to further dissect into possible systems level controls. However, in the wake of upcoming experimental evidences of *FoxM1* and APC^{Cdh1} related G2 delay, it would be very interesting to dissect a reduced G2-M model at systems level to understand *FoxM1* control of G2 transcription and its interplay with APC^{Cdh1} to control G2 delay. Thus, we suggest Cdh1 and *FoxM1* to be good model systems to study G2 delay in mammalian cells.

Implications of liver regeneration to human disease

In humans, liver regeneration occurs most frequently after liver damage by ischaemia or hepatitis an inflammation of the liver that is caused by insults such as toxins, viral infection or immune-mediated injury. Therefore, understanding liver regeneration in humans will help explain how the liver responds to toxic damage by alcohol and drug overdose, or infections like viral hepatitis. Humans with certain hepatic conditions, including cirrhosis (fibrosis of the liver), steatosis (fatty liver), and even those conditions that are due to old age, also have impaired liver regeneration that results in increased morbidity and mortality in response to liver transplantation or toxic chemicals. At present, there is little insight into how the molecular pathways that are necessary for regeneration are altered in these disease states and pathophysiological conditions. Greater insights will be required to develop improved pharmacological therapeutics and surgical approaches for the various medical conditions in which robust liver regeneration is needed. Therefore, understanding the molecular bases regenerating liver cells will not only help to define the pathology in human conditions in which liver regeneration is impaired, it will ultimately provide new treatment options for patients with liver damage.

Cell cycle and cancer

The core cyclin dependent regulatory machinery of the cell cycle is quite well understood in yeast. Our model is an attempt in understanding mammalian cell cycle through a damage induced model of regenerating liver cells in mice. The main challenge for the future is to further understand core cell cycle machinery in mammalian cells and put this into larger contexts of cell physiology, and to investigate, for example, how a cell copes with problems at checkpoints, how it responds to environmental changes, why some cells leave the cell cycle and commit suicide, etc.

The core cell cycle module is regulated by several incoming signals and it drives several downstream events. The duty of this central controller is to process the information it receives and decide how to handle DNA replication and nuclear division. Current models use some parameters as incoming signals and can tell how this input determines the timing of cell cycle events. Some models already investigate how the circadian clock and checkpoint signals regulate cell cycle. These models are very detailed either on the cell cycle machinery or on the signaling network, but comprehensive models that incorporate both control systems in detail do not exist yet. Several models are available for pathways that signal to the cell cycle machinery the presence of nutrients, pheromones, stress inducing agents, etc. These could be merged with appropriate cell cycle models to reveal if our current knowledge of the signaling pathway, cell cycle network interactions is indeed complete. Similarly, many other biological pathways like NF-κB and p53 interact with the cell cycle. While computational models also exist for these processes, they still need to be connected to the cell cycle models and to each other.

Another perspective is to step up from the single cell level and simulate how cell-to-cell interactions alter cell proliferation at the tissue level. This requires multi-scale parallel handling of the cell cycle controls within individual cells while simulating their interactions through signaling at the same time. For this problem we need, first of all, reliable cell cycle models for animal cells, desirably specific models of specific cell types, and in addition we need experimental measurements on the signaling between cells. Such detailed models are far in the future, but we already can learn from some models that take steps in this direction.

These steps lead us to the major future goal: to understand how perturbations of the human cell cycle machinery lead to tumor formation. Indeed mathematical modeling of cancer development is another active research field. Predictive cell cycle models embedded into complex tissue models can help us in the future to understand the dynamics of cancer formation.

After completion of the thesis and the manuscript, two papers have been published which support the work done in this thesis. Gerard and Goldbeter (2009) presents another mammalian cell cycle model based on the similar lines of sequential cyclin-cdk activation. However, it is a growth factor induced model demonstrating repetitive cell cycling in the presence of suprathreshold amounts of GF. The main emphasis is on pRb-E2F network during G1 and Cyclin A dependent activation of Cyclin B during mitotic entry. On the contrary, this model presents re-entry of normally quiescent liver cells in to cell cycle after being induced by injury and focuses on Cdh1, FoxM1 and Wee1 dependent regulation of mitotic events. Kapuy et al. (2009) discuss the systems-level positive feedbacks imposed by Cdc25/Wee1 to be responsible for the irreversibility of mitotic exit in mammalian cells.

Appendix

1 Model Equations

The model contains 24 variables and 75 parameters. The 24 variables are the concentrations of the following proteins involved in the cell cycle control: Cyclins D, E, A, and B; the inhibitor CKIs and its complexes with the active cyclin-cdk complexes; the active and inactive forms phosphatase Cdc25 and of the kinase Wee1; the SCF proteasomal ligases involved in the degradation of cyclin D, Cyclin E and CKIs; and the active and inactive forms of the proteins Cdh1 and Cdc20 involved in the degradation of cyclins A and B.

Vm represents mechaelis menten production parameters. *V* is the non-mechaelis menten production parameters. *P* represents phosphorylation parameters. *d* represents degradation parameters. *kon* is the stoichiometric complex association parameter and *koff* is stoichiometric complex dissociation parameter. Subscripts are the corresponding product and superscripts are the inducers. f is the positive feedback strength on *HGF* and k_{a1} is the positive feedback strength on active *Cyclin B*.

2 G1-S model

parameters

$$Vm_{PIC} = 1.54 \quad Km_{PIC}^{PGE} = 0.9 \quad Km_{PIC}^{Dmg} = 0.5$$
$$d_{PIC} = 0.43 \quad V_{PGE}^{PIC} = 0.61 \quad d_{PGE} = 0.006$$
$$Vm_{IEG}^{PIC} = 4.6 \quad Km_{IEG}^{PIC} = 0.7 \quad d_{IEG} = 6.15$$
$$Vm_{PAI}^{PIC} = 46.15 \quad Km_{PAI}^{PIC} = 0.5 \quad d_{PAI} = 6.15$$
$$V_{HGF}^{IEG} = 5.06 \quad Km_{HGF}^{IEG} = 0.12 \quad Km_{HGF}^{PAI} = 0.18$$
$$d_{HGF} = 0.06 \quad Vm_{HBEGF} = 15 \quad Km_{HBEGF}^{Dmg} = 0.9$$
$$Km_{HBEGF}^{IEG} = 0.005 \quad Km_{HBEGF}^{HGF} = 1 \quad d_{HBEGF} = 0.05$$
$$Vm_{CKI}^{PIC} = 7.69 \quad km_{CKI}^{PIC} = 0.001 \quad d_{CKI} = 7.69$$
$$V_{CD}^{HGF} = 3.12 \quad Km_{CD}^{IEG} = 0.001 \quad d_{CD} = 0.031$$
$$Vm_{CE}^{HBEGF} = 15 \quad Km_{CE}^{HBEGF} = 30 \quad Vm_{CE}^{CD} = 15$$
$$Km_{CE}^{CD} = 30 \quad d_{CE} = 0.02 \quad kon_{CKI}^{CD} = 0.008$$
$$koff_{CDCKI} = 0.0008 \quad kon_{CKI}^{CE} = 2.23 \quad koff_{CECKI} = 6.15$$
$$V_{SCF}^{CE} = 0.77 \quad d_{SCF} \quad = 0.008$$

G1-S model equations

Input signal for the model is a decaying damage (*Dmg*) observed in regenerating hepatocytes after PH. Damage signals induce *priming* via cytokine signaling which further induces growth factor pathways leading to mitogenic stimulation of Cyclin D. Growth factor *HB-EGF* is also directly induced by *Dmg*. Cyclin E is induced both by direct growth factor activation (via *HB-EGF*) and cytokine mediated growth factor activation (via *HGF* and *Cyclin D*. Assumptions and simplifications made for the model equations can be found in the supplementary data from Chauhan et al. (2008).

$$[Dmg] = I_0 \cdot e^{-I_{decay}} \cdot t$$

Priming: Cytokine induced

$$\frac{d[PIC]}{dt} = \frac{Vm_{PIC}}{1+(\frac{PGE}{Km_{PIC}^{PGE}})^2} \cdot \frac{Dmg}{Km_{PIC}^{Dmg}+Dmg} - d_{PIC} \cdot PIC$$

$$\frac{d[PGE]}{dt} = V_{PGE}^{PIC} \cdot PIC - d_{PGE} \cdot PGE$$

$$\frac{d[IEG]}{dt} = \frac{Vm_{IEG}^{PIC} \cdot PIC^3}{{Km_{IEG}^{PIC}}^3 + PIC^3} - d_{IEG} \cdot IEG$$

$$\frac{d[PAI]}{dt} = \frac{Vm_{PAI}^{PIC} \cdot PIC^3}{({Km_{PAI}^{PIC}}^3 + PIC^3} - d_{PAI} \cdot PAI$$

G1 : Growth factor induced

$$\frac{d[HGF]}{dt} = \frac{V_{HGF}^{IEG} \cdot IEG}{1+(\frac{PAI}{Km_{HGF}^{PAI}})^4} \cdot \frac{1+f \cdot HGF}{Km_{HGF}^{IEG}+HGF} - d_{HGF} \cdot HGF$$

$$\frac{d[HBEGF]}{dt} = \frac{Vm_{HBEGF} \cdot Dmg^4}{Dmg^4 + {Km_{HBEGF}^{Dmg}}^4} \cdot \frac{IEG}{Km_{HBEGF}^{IEG}+IEG} \cdot \frac{HGF}{Km_{HBEGF}^{HGF}+HGF} - d_{HBEGF} \cdot HBEGF$$

G1/S
Cyclin D

$$\frac{d[CycD]}{dt} = \frac{k_{CycD}^{HGF} \cdot HGF \cdot IEG}{Km_{CycD}^{IEG}+IEG} - d_{CycD} \cdot CycD \cdot SCF - \frac{d[CycD|CKI]}{dt}$$

$$\frac{d[CycD|CKI]}{dt} = k_{CKI}^{CycD} \cdot CycD \cdot CKI - koff_{CycD|CKI} \cdot CycD|CKI$$

Cyclin E

$$\frac{d[CycE]}{dt} = \frac{Vm_{CycE}^{HBEGF} \cdot HBEGF^2}{HBEGF^2 + Km_{CycE}^{HBEGF^2}} + \frac{Vm_{CycE}^{CycD} \cdot CycD^4}{Km_{CycE}^{CycD^4} + CycD^4} - d_{CycE} \cdot CycE \cdot SCF - \frac{d[CycE|CKI]}{dt}$$

$$\frac{d[CycE|CKI]}{dt} = k_{CKI}^{CycE} \cdot CycE \cdot CKI - koff_{CycE|CKI} \cdot CycE|CKI$$

3 G1/S model extension

The G1/S model was further extended and connected to mitosis via E2F and Cdh1. Sequential activation of E2F by G1-S cyclins – Cyclin D and Cyclin E, leads to transcription of mitotic cyclins – Cyclin A and Cyclin B. At the end of mitosis, Cyclin B degradation leads to Cdh1 binding to APC and activation of APC^{Cdh1} which degrades G1 substrates – SCF and CKI and also the mitotic substrates – Cyclin A, Cyclin B and Cdc20.

E2F–Rb system parameters

$$
\begin{aligned}
E2F_T &= 0.05 & Rb_T &= 1 & p1 &= 3 \\
p_{-1} &= 1 & p_2 &= 4 & p_{-2} &= 1 \\
k_1 &= 100 & off_{RbE2F} &= 4 & k_2 &= 1 \\
off_{RbPE2F} &= 1 & k_3 &= 1 & off_{RbPPE2F} &= 10
\end{aligned}
$$

E2F–Rb equations

The scheme depicts initiation of Rb phosphorylation by Cyclin D and release of some E2F, which then increases Cyclin E transcription. Cyclin E further phosphorylates RbP to release more E2F.

$$
\mathrm{Rb} \underset{p_{-1}}{\overset{CycD \downarrow\, p_1}{\rightleftharpoons}} Rb_p \underset{p_{-2}}{\overset{CycE \downarrow\, p_2}{\rightleftharpoons}} Rb_{pp}
$$

$$
\begin{aligned}
Rb + E2F &\underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E2FRb \\
Rb_p + E2F &\underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E2FRb_p \\
Rb_{pp} + E2F &\underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E2FRb_{pp}
\end{aligned}
$$

Steady state Pool of E2F can be described by the temporal evolution of Cyclin D and Cyclin E, if phosphorylation/dephsophorylation reactions of Rb and association/dissociation of its three phosphorylated forms with E2F is in equilibrium is fast enough, as described in the kinetic equations part of section 6.1.

$$
\begin{aligned}
[Rb_p] &= \frac{p_1}{p_{-1}} \cdot [Rb] \cdot [CycD] \\
[Rb_{pp}] &= \frac{p_1 p_2}{p_{-1} p_{-2}} \cdot [Rb] \cdot [CycD] \cdot [CycE] \\
[Rb \cdot E2F] &= \frac{k_1}{k_{-1}} \cdot [Rb] \cdot [E2F] \\
[Rb_p \cdot E2F] &= \frac{p_1 k_2}{p_{-1} k_{-2}} \cdot [Rb] \cdot [CycD] \cdot [E2F] \\
[Rb_{pp} \cdot E2F] &= \frac{p_1 p_2 k_3}{p_{-1} p_{-2} k_{-3}} \cdot [Rb] \cdot [CycD] \cdot [CycE] \cdot [E2F]
\end{aligned}
$$

Total concentrations

$$
\begin{aligned}
[Rb_T] &= [Rb] + [Rb_p] + [Rb_{pp}] + [Rb \cdot E2F] + [Rb_p \cdot E2F] + [Rb_{pp} \cdot E2F] \\
[E2F_T] &= [E2F] + [Rb \cdot E2F] + [Rb_p \cdot E2F] + [Rb_{pp} \cdot E2F]
\end{aligned}
$$

Substituting the steady state values of $[Rb]$, $[Rb_p]$, $[Rb_{pp}]$, $[Rb \cdot E2F]$, $[Rb_p \cdot E2F]$, and $[Rb_{pp} \cdot E2F]$ into the total concentrations of $[Rb_T]$ and $[E2F_T]$, we solve the equation for

E2F as,

$$
\begin{aligned}
nom &= 1 \\
&\quad + \frac{p1}{p_{-1}} \cdot [CycD] \\
&\quad + \frac{p1 \cdot p_2}{p_{-1} \cdot p_{-2}} \cdot [CycD] \cdot [CycE] \\
denom &= \frac{k_1}{k_{-1}} \\
&\quad + \frac{k_2}{k_{-2}} \cdot \frac{p1}{p_{-1}} \cdot [CycD] \\
&\quad + \frac{k_3}{k_{-3}} \cdot p1 \cdot \frac{p_2}{p_{-1} \cdot p_{-2}} \cdot [CycD] \cdot [CycE] \\
p &= Rb_T - E2F_T + \frac{nom}{denom} \\
q &= -\frac{nom}{denom} \\
E2F &= -p/2 + \sqrt{p \cdot p/4 - q}
\end{aligned}
$$

G1-S model extension parameters

$$
\begin{aligned}
d_{CKI}^{Cdh1} &= 7.69 & k_{SCF}^{Cdh1} &= 0.77 \\
V_{CA}^{E2F} &= 0.25 & V_{CB}^{E2F} &= 1
\end{aligned}
$$

G1-S model extension equations

Following are the equations connecting CKI and SCF from G1-S model to mitosis via APC^{Cdh1}. Transcriptional activation of mitotic cyclins – Cyclin A and Cyclin B by G1-S activated E2F is depicted in mitosis equations in section 4. Also Cdh1 binding and activation of APC^{Cdh1} is explained in mitosis section 4.

$$
\begin{aligned}
\frac{d[CKI]}{dt} &= Vm_{CKI}^{PIC} \cdot \frac{PIC}{km_{CKI}^{PIC} + PIC} - d_{CKI} \cdot CKI - d_{CKI}^{Cdh1} \cdot APCCdh1 \cdot CKI \\
&\quad - \frac{d[CycD|CKI]}{dt} - \frac{d[CycE|CKI]}{dt} - \frac{d[CycD|CKI]}{dt} \\
\frac{d[SCF]}{dt} &= V_{SCF}^{CE} \cdot CycE - d_{SCF}^{Cdh1} \cdot SCF \cdot APCCdh1 - d_{SCF} \cdot SCF
\end{aligned}
$$

4 Mitosis model

Mitosis model parameters

$APC_T = 1$	$d_{CA}^{Cdh1} = 0.5$	
$d_{CA} = 0.05$	$d_{CB}^{Cdh1} = 2$	$d_{CB}^{Cdc20} = 4$
$d_{CB} = 0.1$	$P_{CB}^{Wee1} = 0.8$	$k_1 = 5.4$
$k_2 = 5$	$k_{a1} = 0.75$	$Wee1_b = 0.1$
$P_{Wee1}^{CB} = 100$	$d_{Wee1} = 0.25$	$dphos_{Wee1} = 0.05$
$Cdc25_b = 0.1$	$P_{Cdc25}^{CB} = 50$	$d_{Cdc25} = 0.5$
$dphos_{Cdc25} = 1$	$Cdh1_b = 0.77$	$P_{Cdh1}^{CA} = 0.38$
$P_{Cdh1}^{CB} = 0.77$	$k_{APCCdh1} = 0.5$	$d_{Cdh1} = 0.38$
$dphos_{Cdh1} = 0.05$	$Cdc20_b = 0.04$	$d_{Cdc20} = 0.06$
$P_{Cdc20}^{CB} = 7.7$	$k_{APC}^{Cdc20_p} = 1$	$dphos_{Cdc20} = 0.03$
$d_{Cdc20}^{Cdh1} = 0.77$	$kon_{CA}^{CKI} = 0.01$	$koff_{CACKI} = 0.1$

Cyclin B derivations

Cyclin B exists in two forms in our model: $CycB$ (active Cyclin B) and $CycB_p$ (inactive Cyclin B). Inactive Cyclin B equation is adapted from (Pomerening et al., 2005) with the simplification that the phosphorylation/dephosphorylation of the three inactive forms of Cyclin B *viz* transcribed Cyclin B ($CycB$), Tyrosine phosphorylated ($CycB^Y$), and Tyrosine and Threonine phosphorylated ($CycB^{YT}$) is fast enough to be always in equilibrium; so that equation for $CycB^{YT}$ can be algebraically solved.

$$CycB \underset{Cdc25_p}{\overset{Wee1}{\rightleftharpoons}} CycB^Y \overset{Wee1}{\rightleftharpoons} CycB^{YT} \underset{Wee1}{\overset{Cdc25_p}{\rightleftharpoons}} CycB^T$$

$$CycB = CycB^T\,(active)$$

$$CycB_p = CycB + CycB^Y + CycB^{YT}\,(inactive)$$

$$CycB^Y = k1\frac{1}{Wee1} \cdot CycB^{YT}$$

$$CycB = k2 \cdot \frac{Cdc25_p}{Wee1} \cdot CycB^Y$$

Solving for $CycB^{YT}$

$$CycB^{YT} = \frac{CycB_p \cdot Wee1^2}{Wee1^2 + k1 \cdot Wee1 + k1 \cdot k2 \cdot Cdc25_p}$$

$$CycB = ka1 \cdot Cdc25_p \cdot CycB^{YT}$$

$$CycB_p = P_{CB}^{Wee1} \cdot Wee1 \cdot CycB$$

Mitosis model equations

Assumptions and simplifications made for rest of the model equations are explained in the kinetic equations part of section 6.1. G1-S phase induced E2F activation is explained in previous section 3

Cyclin A

$$\frac{d\,[CycA]}{dt} = Vm_{CycA}^{E2F} \cdot E2F - k_{CycA}^{Cdh1} \cdot [APC|Cdh1] \cdot [CycA] - d_{CycA} \cdot [CycA] - \frac{d\,[CycA|CKI]}{dt}$$

$$\frac{d\,[CycA|CKI]}{dt} = kon_{CycA|CKI} \cdot [CycA] \cdot [CKI] - koff_{CycA|CKI} \cdot [CycA|CKI]$$

Cyclin B

$$
\begin{aligned}
CyclinB(inactive) &= CycB + CycB^{Y} + CycB^{YT} \\
\frac{d\,[CycB_p]}{dt} &= Vm^{E2F}_{CycB} \cdot E2F \\
&\quad - \frac{ka1 \cdot Cdc25_p \cdot [CycB_p] \cdot [Wee1]^2}{[Wee1]^2 + k1 \cdot [Wee1] + k1 \cdot k2 \cdot [Cdc25_p]} \\
&\quad + k \cdot [Wee1] \cdot [cycB] \\
&\quad - k^{Cdh1}_{CycB} \cdot [APC|Cdh1] \cdot [CycB_p] - k^{Cdc20}_{CycB} \cdot [APC|Cdc20_p] \cdot [CycB_p] \\
&\quad - d_{CycB} \cdot [CycB_p] \\
CyclinB(active) &= CycB^{T} \\
\frac{d\,[CycB]}{dt} &= -k \cdot [Wee1] \cdot [CycB] \\
&\quad + \frac{ka1 \cdot [Cdc25_p] \cdot [CycB_p] \cdot [Wee1]^2}{[Wee1]^2 + k1 \cdot [Wee1] + k1 \cdot k2 \cdot [cdc25S]} \\
&\quad - k^{Cdh1}_{CycB} \cdot [APC|Cdh1] \cdot [CycB] - k^{Cdc20}_{CycB} \cdot [APC|Cdc20_p] \cdot [CycB] \\
&\quad - d_{CycB} \cdot [CycB]
\end{aligned}
$$

Wee1

$$
\begin{aligned}
&Wee1(active) \\
&\frac{d\,[Wee1]}{dt} = Wee1_b - V^{CycB}_{Wee1} \cdot [CycB] \cdot [Wee1] + dphos_{Wee1} \cdot [Wee1_p] \\
&\quad - d_{Wee1} \cdot [Wee1] \\
&Wee1(inactive) \\
&\frac{d\,[Wee1_p]}{dt} = V^{CycB}_{Wee1} \cdot [CycB] \cdot [Wee1] - dphos_{Wee1} \cdot [Wee1_p] \\
&- d_{Wee1} \cdot [Wee1_p]
\end{aligned}
$$

Cdc25

$$
\begin{aligned}
&Cdc25(inactive)\\
&\frac{d\,[Cdc25]}{dt} = Cdc25_b - V^{CycB}_{Cdc25}\cdot[CycB]\cdot[Cdc25] + dphos_{Cdc25}\cdot[Cdc25_p]\\
&\qquad - d_{Cdc25}\cdot[Cdc25]\\
&Cdc25(active)\\
&\frac{d\,[Cdc25_p]}{dt} = V^{CycB}_{Cdc25}\cdot[CycB]\cdot[Cdc25] - dphos_{Cdc25}\cdot[Cdc25_p]\\
&\qquad - d_{Cdc25}\cdot[Cdc25_p]
\end{aligned}
$$

APC

$$
\begin{aligned}
APCT &= APC + APCCdh1 + APCCdc20_p\\
APC &= \frac{APCT}{1 + k^{Cdh1}_{APC}\cdot[Cdh1] + k^{Cdc20}_{APC}\cdot[Cdc20_p]}\\
APCCdh1 &= k^{Cdh1}_{APC}\cdot[Cdh1]\cdot[APC]\\
APCCdc20_p &= k^{Cdc20}_{APC}\cdot[Cdc20_p]\cdot[APC]\\
&Cdh1(active)\\
&\frac{d\,[Cdh1]}{dt} = Cdh1_b - V^{CycA}_{Cdh1}\cdot[CycA]\cdot[Cdh1] - V^{CycB}_{Cdh1}\cdot[CycB]\cdot[Cdh1]\\
&\qquad + dphos_Cdh1\cdot[Cdh1_p] - d_{Cdh1}\cdot[Cdh1]\\
&Cdh1(inactive)\\
&\frac{d\,[Cdh1_p]}{dt} = V^{CycA}_{Cdh1}\cdot[CycA]\cdot[Cdh1] - V^{CycB}_{Cdh1}\cdot[CycB]\cdot[Cdh1]\\
&\qquad - dphos_Cdh1\cdot[Cdh1_p] - d_{Cdh1}\cdot[Cdh1_p]\\
&Cdc20(inactive)\\
&\frac{d\,[Cdc20]}{dt} = Cdc20_b - V^{CycB}_{Cdc20}\cdot[CycB]\cdot[Cdc20] + dphos_{Cdc20}\cdot[Cdc20_p]\\
&\qquad - d^{Cdh1}_{Cdc20}\cdot[APC|Cdh1]\cdot[Cdc20] - d_{Cdc20}\cdot[Cdc20]\\
&Cdc20(active)\\
&\frac{d\,[Cdc20_p]}{dt} = V^{CycB}_{Cdc20}\cdot[CycB]\cdot[Cdc20] - dphos_{Cdc20}\cdot[Cdc20_p]\\
&\qquad - d^{Cdh1}_{Cdc20}\cdot[APC|Cdh1]\cdot[Cdc20_p] - d_{Cdc20}\cdot[Cdc20_p]
\end{aligned}
$$

Initial conditions

$$x0_{Wee1} = 0.4 \qquad x0_{Cdh1} = 1.7 \qquad x0_{others} = 0.00001$$

Double peak parameters

Following are the parameters associated with increased feedback strengths of Cdc25, Wee1 and APC^{Cdc20} which lead to a secondary peak in mitosis as observed in regenerating hepatocytes.

$$k_{a1} = 20 \qquad k_{APC}^{Cdc20} = 100$$

5 Mutant parameters

FoxM1 mutant

siRNA mutant of Cyclin A and Cyclin B affecting FoxM1 transcription.

$$V_{CA}^{E2F} = 1 \qquad V_{CB}^{E2F} = 0.1$$

Wee1 mutant

Wee1 mutant for

$$k_{a1} = 0.6 \qquad d_{Wee1} = 0.025$$

HGF injection

$$\begin{aligned} hgfs &= 0.4 \\ dHGF &= \frac{V_{HGF}^{IEG} \cdot IEG}{1 + (\frac{PAI}{Km_{HGF}^{PAI\ 4}})} \cdot (1 + \frac{f \cdot HGF}{Km_{HGF}^{IEG} + HGF}) \\ &\quad - d_{HGF} \cdot HGF + hgfs \cdot (t < 40) \cdot (t > 36) \end{aligned}$$

6 Bistability

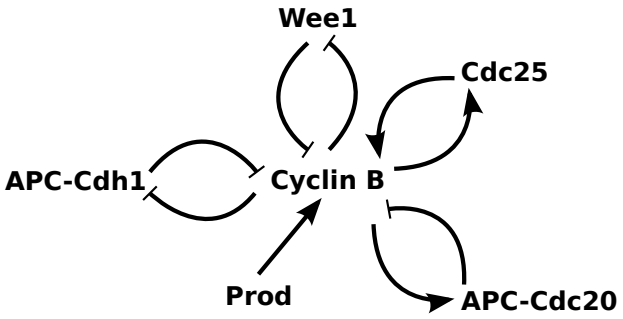

Figure 1: reduced model of mitosis for bistabilty.

A reduced version of model which demonstrates bistability observed in the *Cyclin B* activation during G2-M transition (Fig. 6.17). Total *Cyclin B* levels are dependent on its production and degradation terms. Its activity is enhanced by positive feedbacks from Cdc25 and Wee1 and its degradation is carried out by APC^{Cdh1} in a positive feedback manner and by APC^{Cdc20} in a negative feedback manner (Fig 6.16).

Bistability parameters

$k_1 = 1$	$k_2 = 10$	$k_3 = 10$
$k_4 = 1$	$d_c = 10$	$d_b = 0.05$
$Cdh1_T = 1$	$APC_T = 1$	$Wee1_T = 1$
$Cdc25_T = 1$	$k_7 = 1$	$k_{7i} = 1$
$k_8 = 1$	$Cdc20_t = 1$	$k_9 = 1$
$d_s = 0.1$	$k_5 = 1$	$k_6 = 1$

Initial conditions

$B_T = 0.01$	$APCCdc20s = 0.01$	$prod = 0.4$

Bistability equations

$$
\begin{aligned}
APC_T &= APC + APCCdh1 + APCdc20_p \\
Cdh1_T &= Cdh1 + Cdh1_p + APCCdh1 \\
CycB_T &= CycB + CycB_p \\
Wee1_T &= Wee1 + Wee1_p \\
Cdc25_T &= Cdc25 + Cdc25_p \\
k_1 \cdot [APC] \cdot [Cdh1] &= [APCCdh1] \\
k_2 \cdot [CycB] \cdot [Cdh1] &= [Cdh1_p] \\
k_3 \cdot [Wee1] \cdot [CycB] &= [CycB_p] \\
k_4 \cdot [CycB] \cdot [Wee1] &= [Wee1_p] \\
k_5 \cdot [CycB] \cdot [Cdc25] &= [Cdc25_p] \\
k_6 \cdot [Cdc25_p] \cdot [CycB_p] &= [CycB] \\
k_7 \cdot [APC] \cdot [Cdc20_p] &= [AC20_p] \\
k_8 \cdot [Cdc20] \cdot [CycB] &= [Cdc20_p] \\
APCCdh1 &= AC1 \\
APCCdc20 &= AC20
\end{aligned}
$$

Solving for $[CycB_p]$,

$$CycB_T = CycB + k_3 \cdot \frac{Wee1_T}{1 + k_4 \cdot [CycB]} \cdot [CycB] + \frac{1 + k5 \cdot [CycB]}{k_5 k_6 \cdot [Cdc25_T]}$$

$$CycB = CB$$

$$CycB_T = B_T$$

$$CB = \frac{-b_1 - b_2 \cdot B_T + \sqrt{-4 \cdot b_3 \cdot b_4 + (b_1 + b_2 \cdot B_T)^2}}{2 \cdot b_4}$$

$$Cdc20_p = k_8 \cdot CB \cdot (Cdc20_t - AC20_p)/(1 + k_9 \cdot CB)$$

$$APC = APC_T - AC1 - AC20_p$$

$$AC1 = \frac{1}{2 \cdot k_1} \cdot (k_2 \cdot CB + c_1 - k_1 \cdot AC20_p - \sqrt{(-k_2 \cdot CB - c_1 + k_1 \cdot AC20_p)^2 - 4 \cdot k_1^2 \cdot (c_3 \cdot AC20_p)})$$

$$d[bt]/dt = prod - d_c \cdot AC1 \cdot (B_T - CB) - d_s \cdot AC20_p \cdot (B_T - CB) - d_b \cdot B_T$$

$$d[AC20p]/dt = k7 \cdot APC \cdot Cdc20_p - k7i \cdot AC20p$$

$$d[prod]/dt = 0$$

$$b_1 = -k_4 - k_5 - Cdc25_T \cdot k_5 \cdot k_6 - Cdc25_T \cdot k_3 \cdot k_5 \cdot k_6 \cdot Wee1_T$$

$$b_2 = Cdc25_T \cdot k_4 \cdot k_5 \cdot k_6$$

$$b_3 = 1 - Cdc25_T \cdot B_T \cdot k_5 \cdot k_6$$

$$b_4 = k_4 \cdot k_5 + Cdc25_T \cdot k_4 \cdot k_5 \cdot k_6$$

$$c_1 = k_1 \cdot APC_T + k_1 \cdot Cdh1_T + 1$$

$$c_2 = APC_T \cdot Cdh1_T$$

$$c_3 = c_2 - Cdh1_T$$

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List of Figures

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Selbständigkeitserklärung

Ich erkläre, dass ich die vorliegende Arbeit selbständig und nur unter Verwendung der angegebenen Literatur und Hilfsmittel angefertigt habe.

Berlin, den 28.01.2008 Anuradha Chauhan